

Antagonistic control of cell fates by JNK and p38-MAPK signaling

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During the development and organogenesis of all multicellular organisms, cell fate decisions determine whether cells undergo proliferation, differentiation, or aging. Two independent stress kinase signaling pathways, p38-MAPK, and JNKs, have evolved that relay developmental and environmental cues to determine cell responses. Although multiple stimuli can activate these two stress kinase pathways, the functional interactions and molecular cross-talks between these common second signaling cascades are poorly elucidated. Here we report that JNK and p38-MAPK pathways antagonistically control cellular senescence, oncogenic transformation, and proliferation in primary mouse embryonic fibroblasts (MEFs). Similarly, genetic inactivation of the JNK pathway results in impaired proliferation of fetal hepatoblasts *in vitro* and defective adult liver regeneration *in vivo*, which is rescued by inhibition of the p38-MAPK pathway. Thus, the balance between the two stress-signaling pathways, MKK7-JNK and MKK3/6-p38-MAPK, determines cell fate and links environmental and developmental stress to cell cycle arrest, senescence, oncogenic transformation, and adult tissue regeneration.

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Developmental programs and environmental agents trigger distinct and evolutionarily conserved kinases that relay stress signals mediating proliferation, regeneration, transformation, survival, death, senescence, or cell cycle arrest. The mitogen-activated protein kinases (MAPKs) are a family of kinases that transduce signals from the cell membrane to the nucleus in response to a wide range of stimuli, including stress.^{1–4} Among the MAPK signaling pathways, JNKs and p38-MAPKs were identified to be activated in response to a variety of cellular and environmental stresses such as changes in osmolarity, DNA damage, heat shock, ischemia, cytokines, UV irradiation, or oxidative stress.^{2–4} JNKs are directly activated by the two upstream kinases MKK4 and MKK7. Once activated, JNKs regulate gene transcription via phosphorylation of transcription factors, such as Jun.^{2–5} In contrast, p38-MAPKs are activated by MKK3 and MKK6.^{2,4} These two stress pathways participate in many different intracellular signaling cascades that control a spectrum of cellular processes, including proliferation, differentiation, or apoptosis.

Although JNK and p38-MAPK have the same phylogenetic origin,¹ it is not known why two genetically independent stress-signaling pathways have evolved in evolution. Here we show that the two stress pathways, MKK7-JNK and MKK3/6-p38-MAPK, oppositely control cellular proliferation, regeneration, senescence, and oncogenic transformation, and that the balance between two pathways determines cell fates in response to a variety of stress stimuli.

Results

Antagonistic effect of JNK and p38-MAPK pathways in cell proliferation. Mouse embryonic fibroblasts (MEFs) isolated from *mkk7* mutant embryos display impaired JNK and Jun activation, reduced cell proliferation, premature senescence, as well as augmented susceptibility to stress-induced aging (Figure 1 and Wada *et al.*⁶). In contrast, activation of p38-MAPK can also result in premature senescence of MEFs,⁷ whereas pharmacological p38-MAPK inhibition induces increased cell proliferation.⁸ To assess functional hierarchies and potential molecular cross-talks between the JNK and p38-MAPK stress pathways in cell fate decisions, we tested the effects of p38-MAPK modulation in *mkk7*^{-/-} MEFs. Surprisingly, inhibition of p38-MAPK α/β using the selective inhibitor SB202190 restored the proliferation of *mkk7*-deficient MEFs to that of MKK7-expressing control cells (Figure 1a). To further confirm whether the reversal effects of cell proliferation are indeed p38-MAPK dependent, we introduced dominant-negative forms of MKK3 and MKK6 (MKK3-DN and MKK6-DN) into *mkk7*^{-/-} MEFs to genetically inactivate p38-MAPK activity. Similar to SB202190, both MKK3-DN and MKK6-DN restored cell proliferation in *mkk7*-deficient cells (Figure 1b). Expression of MKK3-DN and MKK6-DN in MEFs inhibited UV-induced p38-MAPK activation as measured by ATF2 phosphorylation, but had no substantial effect on UV-induced JNK activation (Supplementary Figure 1a). Thus, inhibition of

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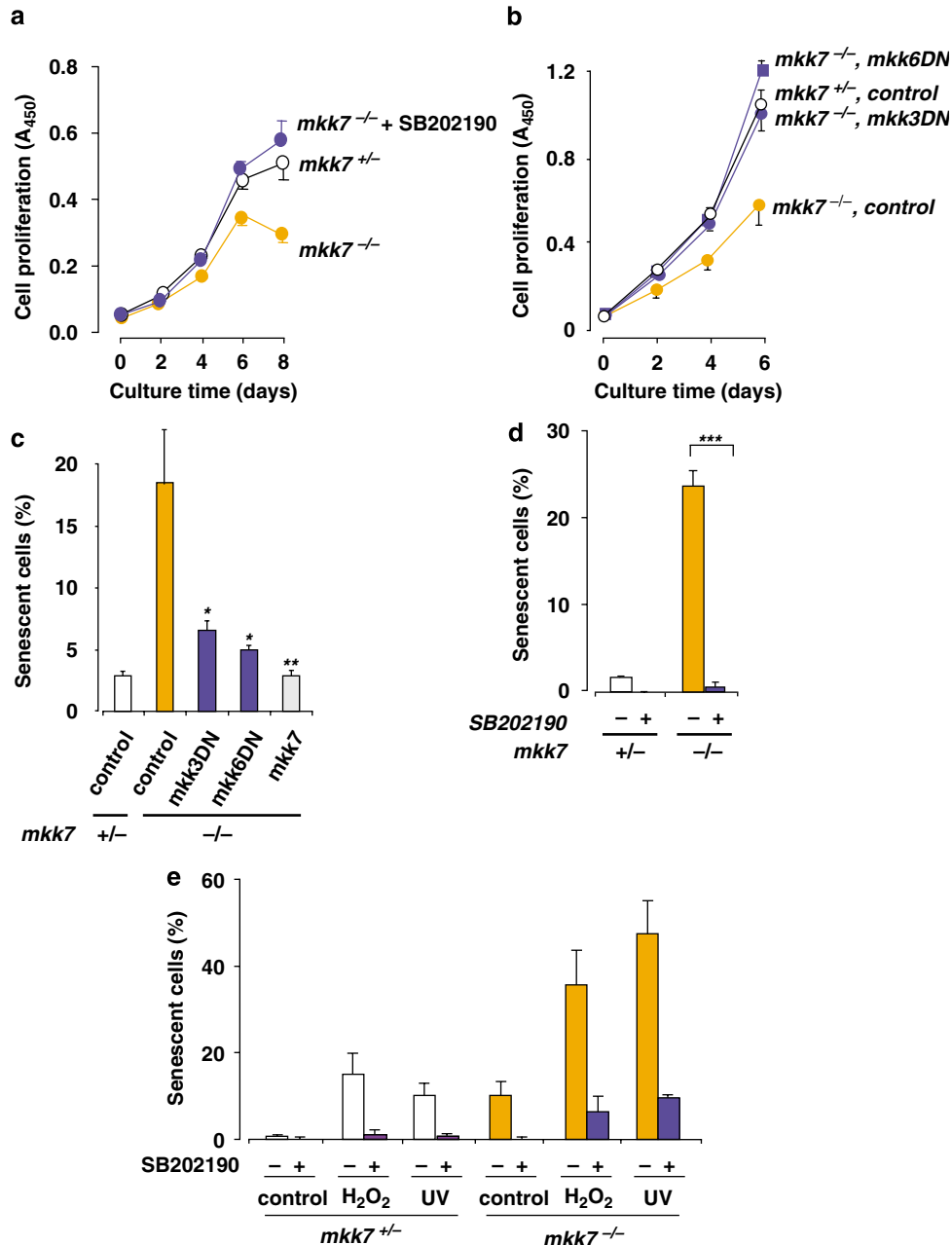


Figure 1 Antagonistic control of cell proliferation and cellular senescence by MKK7-JNK and p38-MAPK signaling. (a) Decreased proliferation of *mkk7*^{-/-} MEFs and rescue of the proliferative defect by the p38-MAPK inhibitor SB202190. Proliferation was determined using the cell proliferation reagent WST-1 at the indicated time points. (b) Expression of dominant-negative MKK3 (MKK3-DN) or MKK6 (MKK6-DN) restores decreased proliferation in *mkk7*^{-/-} MEFs. An empty pBabe vector was used as control. (c, d) The MKK3/6-p38-MAPK pathway antagonizes MKK7-JNK in cellular senescence. Transfection of *mkk7*^{-/-} MEFs with *mkk3DN*, *mkk6DN*, and wild-type *mkk7* as control (c) or treatment of *mkk7*^{+/-} and *mkk7*^{-/-} MEFs with the p38-MAPK α/β inhibitor SB202190 (10 μ M) (d) prevents premature senescence. Mean numbers of SA- β -galactosidase-positive senescent cells are shown. These data were confirmed using classical senescent cell morphology. * $P > 0.05$; ** $P > 0.01$; *** $P > 0.001$ (ANOVA) between control *mkk7*^{-/-} MEFs and *mkk7*^{-/-} MEFs transfected with *mkk3DN*, *mkk6DN*, and *mkk7*, or after treatment with SB202190. (e) Differential roles of the JNK and p38-MAPK stress kinase pathways in environmental stress-induced senescence. *mkk7*^{+/-} and *mkk7*^{-/-} MEFs (passage 3) were UV irradiated (10 J/m²) or treated with H₂O₂ (150 μ M) in the presence or absence of 10 μ M SB202190. Stress-induced senescence (mean numbers of senescent cells determined by enlarged morphology and SA- β -galactosidase) was assayed 3 days later. Data are shown as mean \pm S.E.M.; $n = 3$

the p38-MAPK pathway can rescue the proliferative defect observed in *mkk7*^{-/-} MEFs.

JNK and p38-MAPK antagonistically control cellular senescence. To examine whether these antagonistic

effects are limited to cell proliferation, we examined other MKK7-JNK-dependent cell fate responses such as senescence.⁶ Treatment of *mkk7*^{-/-} MEFs with the selective p38-MAPK α/β inhibitor SB202190, as well as overexpression of MKK3-DN and MKK6-DN indeed

prevented premature senescence (Figure 1c and d). Treatment of wild-type cells with H₂O₂ or UV radiation resulted in premature senescence phenotype that is markedly enhanced in *mkk7*^{-/-} MEFs (Figure 1e). Importantly, inhibition of p38-MAPK α/β abolished premature senescence in both *mkk7*^{+/-} and *mkk7*^{-/-} MEFs following treatment with H₂O₂ and UV (Figure 1e). These results show that MKK7-JNK and MKK3/6-p38-MAPK pathways play opposite roles in multiple cell fate responses: inactivation of the MKK7-JNK pathway results in defective proliferation and premature senescence; in contrast, inhibition of MKK3/6-p38-MAPK pathway triggers cell proliferation and markedly suppresses the cellular aging response. Importantly, inhibition of the p38-MAPK pathway allows *mkk7*-null cells to exit the cell cycle block and rescues *mkk7*^{-/-} MEFs from a premature aging response in response to environmental stress.

The JNK and p38-MAPK stress pathways balance oncogenic transformation. It has been shown that cell proliferation, senescence, and cancer are closely linked at the molecular level.⁹ Both MKK7-JNK-Jun as well as MMK3/MMK6-p38-MAPK signaling have been implicated in multiple cancers in humans.^{2,10} Since MKK7-JNK and MKK3/6-p38-MAPK pathways exhibit antagonistic roles in cell proliferation and senescence, we speculated that both pathways may also exhibit opposing functions in oncogenic transformation. As reported previously,¹¹ MEFs can be transformed by ectopic overexpression of oncogenic Ras^{12V} plus c-myc (Figure 2a and b). Intriguingly, *mkk7*^{-/-} MEFs were markedly resistant against Ras^{12V} and c-myc-induced transformation

(Figure 2a and b). Ectopic expression of MKK7 enhanced colony formation in control MEFs and restored oncogenic transformation of *mkk7*^{-/-} MEFs (Figure 2a and b). p38-MAPK inhibition by MKK6-DN promoted cell transformation in control cells and, most importantly, in *mkk7*^{-/-} MEFs (Figure 2a and b). In all our experiments p38-MAPK inhibition in *mkk7*^{-/-} MEFs never reached transformation efficacies of MKK6-DN-transfected control MEFs or MKK7-transfected control and *mkk7*^{-/-} MEFs. To extend these findings to another oncogenic cancer model, we examined the involvement of the MKK7-JNK and p38-MAPK pathways in Tpr-Met-induced cell transformation.¹² Similar to Ras^{12V} and c-myc, overexpression of oncogenic Tpr-Met triggered transformation in control MEFs, but to a much lesser extent in *mkk7*-deficient cells. Importantly, suppression of the p38-MAPK via MKK6-DN again promoted cell transformation in both control MEFs and restored oncogenic transformation in *mkk7*-deficient MEFs (Figure 2c). These results using different oncogenes show that the MKK7-JNK pathway is critical for cellular transformation, whereas p38-MAPK signaling negatively regulates oncogene-induced cellular transformation.

CDC2 is a potential target for molecular antagonisms. How can activation of two different cellular stress pathways result in opposite effects on cell cycle progression, senescence, or transformation in MEFs? We have recently identified the cell cycle kinase CDC2 as a molecular target for the MKK7-JNK signaling pathway in MEFs that links environmental stress and developmental cues to cell proliferation and senescence.⁶ Biochemically, CDC2 has also been identified as a downstream target for the p38-MAPK pathway.¹³ We therefore speculated that a molecular antagonism between these two different stress-signaling pathways might converge on CDC2. Since *mkk7*-deficient MEFs show reduced CDC2 expression and impaired CyclinB1 associated CDC2 kinase activity depending on the cell passage,⁶ we examined whether CyclinB1 associated CDC2 kinase activity can be rescued by inhibition of the p38-MAPK pathway. Whereas CDC2 expression and total CDC2 kinase activity were indeed markedly reduced in *mkk7*-null MEFs, inhibition of p38-MAPK resulted in increased CDC2 activity, without obvious effects on CDC2 expression (Figure 3a). Inhibition of p38-MAPK also resulted in increased CDC2 activity in *mkk7*^{+/-} MEFs, suggesting that p38-MAPK can repress CDC2 activation even in the presence of MKK7 expression (Supplementary Figure 1b). In the same experimental system, we did not observe substantially altered CyclinD1-associated Cdk4 activity (Figure 3b). These results suggest that antagonistic regulation of CDC2 by MKK7-JNK and MKK3/6-p38 may be one of checkpoints by which different stress-signaling pathways control cell fate in MEFs.

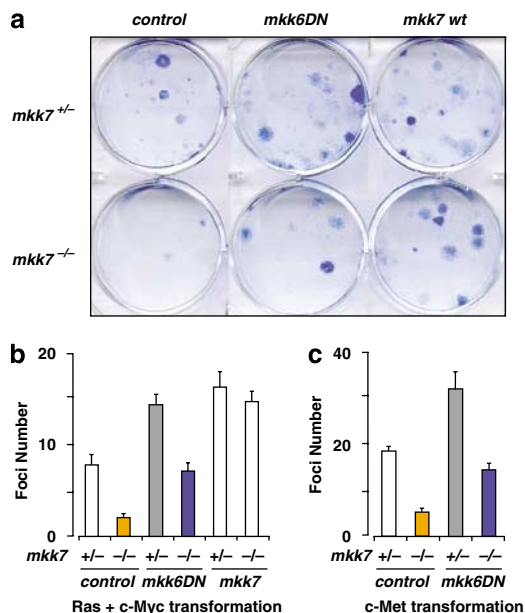


Figure 2 Opposing effect of the two stress pathways on oncogene-mediated cellular transformation. (a–c) Primary, early-passage control and *mkk7*^{-/-} MEFs were transformed with Ras^{12V} plus c-Myc or Tpr-Met in the presence or absence of MKK6-DN. Foci formation was determined on day 21. Representative appearance of foci induced by Ras^{12V} and c-Myc (a) and average foci numbers in Ras^{12V}- and c-Myc- (b), and Tpr-Met (c)-induced transformation are shown. All data are shown as mean \pm S.E.M.; n = 3

Antagonism of the JNK and p38-MAPK pathways in hepatocyte proliferation and liver regeneration *in vivo*. To determine whether the observed molecular antagonism between MKK7-JNK and p38-MAPK pathways also occurs in other cell types, we analyzed mouse embryonic hepatoblasts. Inactivation of MKK7 in mice leads to embryonic lethality due to defective fetal liver

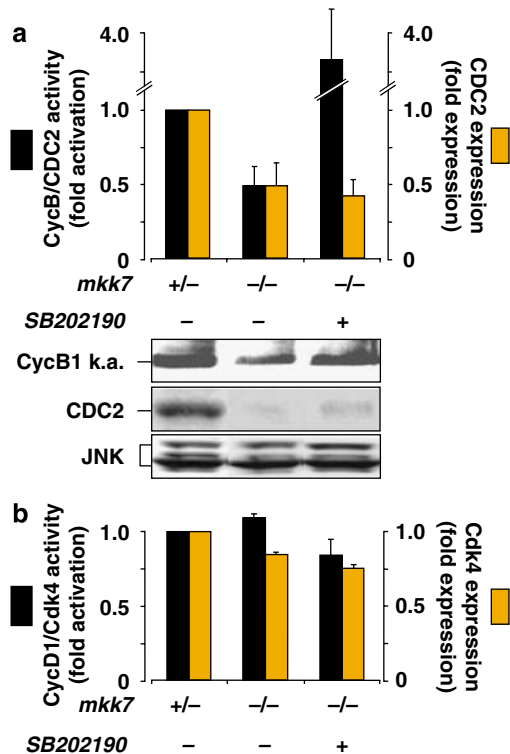


Figure 3 JNK and p38-MAPK differently control the cell cycle regulator CDC2 in MEFs. (a, b) CyclinB1-associated CDC2, but not CyclinD1-associated Cdk4 kinase activities (k.a.) are enhanced by inhibition of p38-MAPK α/β with SB202190 (10 μ M) in *mkk7*^{-/-} MEFs. (a) Decreased CyclinB1 (CycB1)-associated CDC2 kinase activities in *mkk7*^{-/-} MEFs were enhanced by inhibition of p38-MAPK α/β . CDC2 kinase activity data were normalized to protein expression. Data are shown as mean \pm S.E.M.; $n = 4$. (b) CyclinD1 (CycD1)-associated Cdk4 kinase activities were not significantly affected in *mkk7*^{-/-} MEFs in the absence or presence of the p38-MAPK α/β inhibitor SB202190. Expression of Cdk4 and CDC2 was determined by Western blot. Data are shown as mean \pm S.E.M.; $n = 3$

formation around embryonic days 11.5–13.5 (Wada *et al.*⁶) and impaired hepatoblast proliferation (Figure 4a). Inhibition of p38-MAPK α/β enhanced hepatoblast proliferation from control mice and rescued the impaired proliferation of *mkk7*^{-/-} hepatoblasts (Figure 4a). To determine whether functional antagonism between the MKK7-JNK and p38-MAPK pathways is limited to embryonic cells, that is, MEFs and hepatoblasts, or if such antagonism is also operational in adult tissue, liver regeneration (as stress) after partial hepatectomy was examined. Partial hepatectomy in wild-type animals triggers liver regeneration due to rapid re-entry of hepatocytes into the cell cycle, as determined by Ki67 staining (Figure 4b). Jun is a critical downstream target of MKK7-JNK: overexpression of Jun is sufficient to rescue defective proliferation and premature senescence in *mkk7*^{-/-} MEFs,⁶ and inactivation of *jun* in adult mouse hepatocytes using *Mx-Cre Jun*^{fllox} alleles results in defective hepatocyte proliferation (Figure 4b and Behrens *et al.*¹⁴). Since p38-MAPK α is the predominant isoform expressed in liver (<http://www.genecards.org>), we therefore generated adult mice with conditional deletion of both Jun and p38-MAPK α (*Mx-Cre;Jun*^{fllox/fllox}*p38-MAPK α* ^{fllox/fllox}) in hepatocytes. Intriguingly, *in vivo* hepatocyte proliferation was rescued in

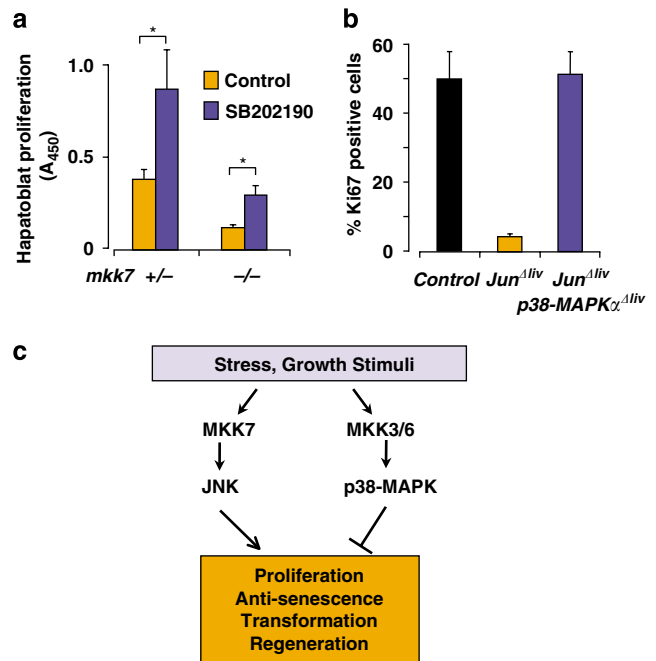


Figure 4 The JNK and p38-MAPK inversely regulate liver regeneration *in vivo*. (a) Inhibition of p38-MAPK α/β with SB202190 (10 μ M) increases proliferation of *mkk7*^{+/-} and *mkk7*^{-/-} hepatoblasts. Embryonic hepatoblast (3×10^3 cells/well) were isolated from E11.5 *mkk7*^{+/-} and *mkk7*^{-/-} littermate embryos and proliferation was determined as in Figure 1a and b. Data are shown as mean \pm S.E.M.; $n = 3$. * $P > 0.05$ (Student's *t*-test) (b) Genetic inactivation of p38-MAPK α restores hepatocyte proliferation in *jun*-deficient livers after partial hepatectomy. Hepatocyte proliferation in control, *Jun*^{Aliv}, and *Jun*^{Aliv} *p38-MAPK α* ^{Aliv} mice was determined by immunodetection of the proliferation marker Ki67 48 h after partial hepatectomy. Data are shown as mean percentages of Ki67-positive hepatocytes \pm S.E.M.; $n = 3$. (c) Opposing effects of the MKK7-JNK and p38-MAPK stress pathways in multiple cell fate responses. Signaling via the MKK7-JNK-Jun pathway promotes cell proliferation, transformation, liver regeneration, and prevents cellular senescence. In contrast, the MKK3/6-p38-MAPK pathway suppresses these cell fate responses and antagonizes the JNK pathway

mice lacking both *jun* and *p38-MAPK α* (Figure 4b). We next analyzed CyclinB1-associated CDC2 kinase activity. Genetic deletion of both *jun* and *p38-MAPK α* resulted in strong induction of CyclinB1-associated kinase activity following partial hepatectomy (Supplementary Figure 2a and b). Of note, previous experiments have also reported reduced CyclinB1-associated kinase activity in *jun* mutant mice.¹⁴ Consistent among all samples analyzed, markedly enhanced CyclinB1-associated CDC2 kinase activity was observed in the absence of p38-MAPK (Supplementary Figure 2, and not shown). These results show that the two stress pathways exert antagonistic effects on cell fates in embryonic and adult tissues and multiple cell types.

Discussion

Our data indicate that the two distinct stress-signaling pathways, MKK3/MKK6-p38-MAPK *versus* MKK7-JNK, can exert opposite effects on cell cycle progression, cellular senescence, oncogenic transformation, and adult liver regeneration (Figure 4c). In response to environmental stress and oncogenic signals, the MKK7-JNK pathway promotes

proliferation, transformation, and regeneration and prevents premature senescence. By contrast, activation of MKK3/6-p38-MAPK pathway antagonizes these MKK7-JNK functions. Although the roles of each MKK3/6-p38-MAPK and MKK7-JNK pathway in cell proliferation and senescence have been previously shown by us and other groups,^{6–8} the molecular cross-talks and the balance of the both pathways are not well described. Our data in fibroblasts and hepatocytes suggest that the two distinct stress-signaling pathways can both act on CDC2 with opposite effects. Regulation of CDC2 by MKK7-JNK and MKK3/6-p38 might be one mechanism by which stress signaling is coupled to cancer, cell cycle progression, or senescence. However, antagonistic cell fate regulations by the two stress pathways most likely also includes mechanisms other than CDC2, since many genes and proteins are regulated by MKK7-JNK-Jun and MKK3/6-p38-MAPKs.²

In conclusion, our results show that the stress kinase p38-MAPK pathway antagonizes MKK7-JNK functions in terms of cellular proliferation, senescence, oncogenic transformation, and liver regeneration. These data indicate that the balance between MKK7-JNK and MKK3/6-p38-MAPK signaling determines cell fate decisions in response to environmental and developmental stress in cancer, aging, and tissue regeneration.

Materials and Methods

Mice. *mkk7*-deficient MEFs were obtained as described.⁶ To disrupt the murine Jun and p38-MAPK α in liver, mice carrying *jun*^{lox} (Behrens *et al.*¹⁴) and *p38-MAPK α* ^{lox} (Engel *et al.*¹⁵) alleles were crossed to Mx1-Cre transgenic mice. The Cre transgene was induced in adult mice upon injections of polyinosinic-polycytidylic ribonucleic acid as described previously.¹⁴ Deletions of Jun and p38-MAPK α were confirmed by Western blot using Jun- and p38-MAPK-specific antibodies (Cell Signaling, data not shown). Partial hepatectomy was performed as described previously.¹⁶ Mouse experiments were performed according to institutional guidelines.

MEF cultures. MEFs were prepared from E11.5 embryos and maintained in DMEM with 10% FCS. Since *mkk7*^{+/+} and *mkk7*^{+/-} MEFs behave equally in our experimental systems, *mkk7*^{+/-} MEFs were used as controls. Cell proliferation in MEFs was determined using WST-1 (Roche). HA-tagged dominant-negative forms of MKK3 and MKK6 that carry inactivating AL mutations in their respective phosphorylation sites attenuate p38-MAPK activities, but not JNK activities in the presence of sorbitol stimuli.¹⁷ Dominant-negative MKK3 and MKK6 were ligated into a pBabe-puro vector to generate the pBabe-puro-MKK3DN and pBabe-puro-MKK6DN expression constructs. The expression of MKK3DN and MKK6DN was confirmed using an anti-HA antibody (Sigma; Supplementary Figure 1a). For ectopic gene expression in MEFs, the retroviral vectors pBabe-puro as control, pBabe-puro-MKK7⁶, pBabe-puro-MKK3DN, or pBabe-puro-MKK6DN were transfected into phoenix-E packaging cells. MEFs were cultured in the supernatant of the packaging cells containing 5 μ g/ml polybrene for 1 day and selected with 2.5 μ g/ml puromycin for 2 days followed by 2 days of recovery. Empty pBabe-puro as control vector did not show any detectable effect on the phenotype of MEFs. SB202190 (Calbiochem) or the solvent DMSO were added into cultures as indicated in the figure legends. If not stated otherwise, MEFs at passages 4–5 were used in all experiments. To detect senescent cells, SA- β -gal staining was performed (Cell Signaling). It should be noted that all SA- β -gal-positive cells had the typical flattened cell morphology characteristic of senescent cells.¹⁸

Oncogenic transformation. MEFs were transformed with oncogenic Ras-V12 plus c-Myc or with oncogenic Tpr-Met using the retroviral vector pBabe as described above. Three thousand cells at passage 4 were plated on six-well plates and cultured for 21 days. The resultant foci were stained with Coomassie brilliant blue, and the numbers of foci were determined.

Kinase assays. To detect Cyclin-associated kinase activities, liver samples or MEFs (passages 2–4) were homogenized in lysis buffer containing 50 mM Tris-HCl (7.5), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% Tween-20, 10% glycerol, 1 mM NaF, 0.2 mM Na₃VO₄, and protease inhibitor cocktail (Roche), and CyclinD1 and CyclinB1 were immunoprecipitated from the supernatants at 4°C using specific antibodies (Santa Cruz). Kinase activities were determined using GST-Rb for CyclinD1 (Santa Cruz) and HistoneH1 for CyclinB1 (Roche) as substrates in the presence of [γ -³²P]ATP. To detect JNK and p38-MAPK kinase activities, JNK and p38-MAPK were immunoprecipitated from MEF lysates using specific antibodies (Santa Cruz and Cell Signaling, respectively). Kinase activities were determined using GST-Jun and GST-ATF2 as substrates, in the presence of [γ -³²P]ATP. Protein expression levels were determined using antibodies specific to CyclinB1, CyclinD1, Cdk4 (Santa Cruz), JNK, CDC2 (Cell Signaling), and actin (Sigma).

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Competing financial interests

The authors declare that there is no competing financial interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)