CHAPTER 3

Mapkapk3 is a suppressor of anchorage-independent growth

Tinke L. Vormer, Camiel L.C. Wielders, Marije Scholte and Hein te Riele

Manuscript in preparation
Mapkapk3 suppresses anchorage-independent growth

Abstract
Ablation of the pocket proteins pRB, p107 and p130 in mouse embryonic fibroblasts (MEFs) abrogated RAS<sup>V12</sup>-induced senescence, but was not sufficient to support RAS<sup>V12</sup>-induced transformation. To identify events that support transformation, we performed an insertional mutagenesis screen in RAS<sup>V12</sup>/Rb<sup>/−</sup>p130<sup>/−</sup>p107<sup>/−</sup> MEFs. As insertional mutagen we used the ERM vector, which was specifically designed to enhance gene function, and scored for colony formation in soft agar. In one of the colonies, we identified an ERM integration in Mapkapk3, however, this integration appeared to abrogate rather than to enhance Mapkapk3 expression. Consistently, we found that shRNA-mediated down regulation of Mapkapk3 or chemical inhibition of the upstream Map kinase p38 promoted anchorage-independent growth.

Introduction
The pocket proteins pRB, p107 and p130 play a key role during cell cycle regulation by promoting the G<sub>0</sub>/G<sub>1</sub> state. Consistently, our previous experiments in mouse embryonic fibroblasts (MEFs) showed that G<sub>1</sub> arrest in response to various growth inhibitory signals was abrogated by loss of either pRB and p130, pRB and p107 or all three pocket proteins simultaneously. E.g., whereas wild-type MEFs entered premature senescence upon expression of RAS<sup>V12</sup>, Rb<sup>/−</sup>p130<sup>/−</sup> (DKO), Rb<sup>/−</sup>p107<sup>/−</sup> (DKO) and Rb<sup>/−</sup>p130<sup>/−</sup>p107<sup>/−</sup> (TKO) MEFs continued to proliferate. Strikingly though, these pocket-protein deficient MEFs could not grow anchorage independently upon expression of RAS<sup>V12</sup>, demonstrating that transformation requires additional events (Vormer et al., 2008; Dannenberg et al., 2004; Peep et al., 2001; Dannenberg et al., 2000).

In Chapter 2, we showed that transformation of RAS<sup>V12</sup>-expressing DKO or TKO MEFs could be achieved by expression of TBX2. The TBX2 transcription factor, which can both activate and repress transcription of a variety of target genes (Paxton et al., 2002; Chen et al., 2001), can downregulate well-known cell cycle inhibitors such as p19<sup>ARF</sup> and, to a lower extent, p16<sup>INK4A</sup> (Lingbeek et al., 2002; Jacobs et al., 2000). As p16<sup>INK4A</sup> functions upstream of the pocket proteins, TBX2-mediated downregulation of p16<sup>INK4A</sup> is unlikely to contribute to the transformation of pocket protein-deficient MEFs. In line with this, we found that downregulation of p19<sup>ARF</sup>, p53 or p21<sup>CIP1</sup> contributed to anchorage-independent growth of RAS<sup>V12</sup>/TKO MEFs, whereas downregulation of p16<sup>INK4A</sup> did not (Vormer et al., 2008). Interestingly, downregulation of p53 more robustly induced soft agar colony formation than downregulation of p21<sup>CIP</sup>, indicating that additional p53 effectors contributed to the induction of transformation. Similarly, one may envision that targets of TBX2 functioning in distinct tumor suppressor pathways could contribute to TBX2-induced transformation of pocket protein-deficient MEFs. We therefore aimed to identify additional inducers of anchorage-independent growth. For this purpose, we performed
an insertional mutagenesis screen in RAS\textsuperscript{V12}/TKO MEFs and found that an inactivating proviral insertion in the \textit{Mapkapk3} (mitogen-activated-protein-kinase-activated protein-kinase 3) gene promoted anchorage-independent growth. Consistently, shRNA-mediated down-regulation of Mapkapk3 induced anchorage-independent growth of RAS\textsuperscript{V12}/pocket protein-deficient MEFs.

\textbf{Results}

\textit{A retroviral insertional mutagenesis screen to identify mediators of anchorage-independent growth}

To identify promoters or inhibitors of anchorage-independent growth, we performed a retroviral insertional mutagenesis screen in RAS\textsuperscript{V12}/TKO MEFs using the Enhanced Retroviral Mutagen (ERM) vector (Liu et al., 2000). This retroviral vector is a derivative of pBABE and was designed to enhance gene transcription upon integration into the genomic DNA. When the ERM virus integrates upstream of an endogenous promoter, transcription can be increased via enhancer elements present in ERM. Additionally, a splice donor site at the 3’ end of ERM enables splicing towards a splice acceptor site of an endogenous exon, resulting in a fusion transcript under the control of the ERM-promoter. The latter can occur when integration occurs either upstream or within a genetic locus. RAS\textsuperscript{V12}/TKO MEFs were infected with ERM, plated under non-adherent conditions (soft agar) and monitored for colony formation. Infection with ERM caused a four-fold increase in the number of soft agar colonies compared to non-infected RAS\textsuperscript{V12}/TKO MEFs. Soft agar colonies produced by ERM/RAS\textsuperscript{V12}/TKO MEFs were isolated and the position of the ERM integration in the genomic DNA was determined using splinkerette PCR. This analysis identified an ERM-integration in intron 2 of \textit{Mapkapk3} in one of the colonies (Fig. 1A). The position of the ERM-integration into \textit{Mapkapk3} was confirmed by PCR analysis using genomic DNA (Fig. 1B) and by RT-PCR analysis. Sequencing of the RT-PCR product revealed that the ERM vector had spliced to exon 3 of \textit{Mapkapk3} but that the ERM-\textit{Mapkapk3} fusion was out of frame (Fig. 1C). This suggested that transformation was not due to increased expression of \textit{Mapkapk3}. To study the effect of this integration, we generated cDNA from the ERM-\textit{Mapkapk3} out-of-frame fusion and inserted this into pBABE. Additionally, full-length wild-type \textit{Mapkapk3} cDNA was inserted into pBABE. Infection of RAS\textsuperscript{V12}/TKO MEFs with these vectors and subsequent soft agar plating showed that pBABE-\textit{Mapkapk3} inhibited the number of background colonies produced by RAS\textsuperscript{V12}/TKO MEFs, whereas pBABE-ERM-\textit{Mapkapk3} had no effect (data not shown). These results suggest that overexpression of Mapkapk3 suppressed anchorage-independent growth and furthermore, that inactivation of one allele of \textit{Mapkapk3} by ERM insertion had induced transformation.
**Mapkapk3 suppresses anchorage-independent growth**

RNAi-mediated downregulation of Mapkapk3 promotes anchorage-independent growth

To verify that downregulation of Mapkapk3 promotes anchorage-independent growth, we generated five different pRetroSuper (pRS) vectors targeting Mapkapk3. Upon infection of RAS\(^{V12}/\)Rb\(^{-/-}\)p107\(^{-/-}\)MEFs, 2 out of 5 pRS-Mapkapk3 vectors induced colony formation in methylcellulose (Fig. 2A). Additionally, we investigated whether downregulation of the p38 MAPK pathway, which is an upstream regulator of Mapkapk3, could support anchorage-independent growth. Indeed, treatment with the p38 inhibitor SB203580 induced colony formation in RAS\(^{V12}/\)Rb\(^{-/-}\)p107\(^{-/-}\) MEFs, cultured in methylcellulose (Fig. 2B). In conclusion, downregulation of p38/Mapkapk3 induced anchorage-independent growth in RAS\(^{V12}/\)pocket protein-deficient MEFs.

---

**Figure 1: An insertional mutagenesis screen for anchorage-independent growth of RAS\(^{V12}/\)TKO MEFs reveals integration of ERM into Mapkapk3.** (A) Genomic organization of the Mapkapk3 locus in a RAS\(^{V12}/\)TKO soft agar colony (number 3.17). ERM is integrated into intron 2 of Mapkapk3. (B) PCR analysis of 3 different soft agar colonies, demonstrating the presence of the ERM-Mapkapk3 fusion in colony 3.17. The positions of the used primers are depicted in (A). (C) Sequence of the ERM-Mapkapk3 RT-PCR product that was derived from colony 3.17. Reverse transcription and amplification of ERM-Mapkapk3 was performed as described in the Materials and Methods section. Red bases represent the last part of the ERM sequence, blue bases represent the out-of-frame Mapkapk3 sequence, which is the resultant of splicing of ERM towards exon 3 of Mapkapk3. After reaching a stop codon, the sequence is shown in black.
Figure 2: Downregulation of Mapkapk3 or p38 MAPK promotes anchorage-independent growth. (A) Rb\(^{-/-}\)p107\(^{-/-}\) MEFs were infected with pBABE-RAS\(^{V12}\) plus pRS-Mapkapk3 or empty pRS and cultured in methylcellulose for 3 weeks. (B) Rb\(^{-/-}\)p107\(^{+/+}\) MEFs were infected with pBABE-RAS\(^{V12}\), treated with the indicated concentrations of the p38 inhibitor SB203580 and subsequently cultured in methylcellulose for 3 weeks. Pictures were taken using a non-phase-contrast lens (2.5x magnification).

**Mapkapk3 regulation by TBX2 and anchorage**

Since TBX2 over-expression induced anchorage-independent growth, we performed micro-array analyses of RAS\(^{V12}/Rb^{-/-}\)p107\(^{-/-}\) MEFs to identify mRNAs that were regulated by loss of anchorage or the presence of TBX2. Strikingly, Mapkapk3 appeared to be one of the regulated genes. Fig. 3A shows that non-adherent RAS\(^{V12}/Rb^{-/-}\)p107\(^{-/-}\) MEFs displayed a two-fold increase in Mapkapk3 mRNA level compared to their adherent counterparts. Expression of TBX2 in non-adherent RAS\(^{V12}/Rb^{-/-}\)p107\(^{-/-}\) MEFs reverted Mapkapk3 expression to the level observed in adherent RAS\(^{V12}/Rb^{-/-}\)p107\(^{-/-}\) MEFs (Fig. 3A). These results suggest that the induction of Mapkapk3 upon loss of anchorage functions as a growth suppressor mechanism that can be abrogated by expression of TBX2. Of note, the related Mapkapk2, which was previously identified as a downregulated TBX2-target (Chen et al., 2001), was not transcriptionally induced upon loss of anchorage, and was only slightly inhibited by TBX2. For comparison, loss of anchorage also caused a two- to three-fold increase in p21\(^{CIP1}\) and p27\(^{KIP1}\) mRNA levels, which could be inhibited by expression of TBX2 (Fig. 3B). This is in line with our previous studies showing that downregulation of p21\(^{CIP1}\) could promote anchorage-independent growth of RAS\(^{V12}/pocket protein-deficient MEFs (Vormer et al., 2008). Taken together, our results identify
**Mapkapk3** as a haploinsufficient suppressor of anchorage-independent growth and suggest that the transforming activity of TBX2 is at least partially mediated by down regulation of **Mapkapk3**.

### Discussion

In this study, we have identified **Mapkapk3** as a suppressor of anchorage-independent proliferation of pocket protein-defective cells. **Mapkapk3** was found to be transcriptionally induced upon anchorage deprivation and downregulation of **Mapkapk3** enabled anchorage-independent growth: inactivation of one allele of **Mapkapk3** by retroviral insertion or shRNA-mediated downregulation of **Mapkapk3** promoted proliferation under non-adherent conditions of RAS$^{V12}$/TKO and RAS$^{V12}$/DKO MEFs. Furthermore, we found that
the oncogene TBX2 can downregulate *Mapkapk3*. Thus, downregulation of *Mapkapk3* is likely to be one of the mechanisms through which TBX2 exerts its transforming activity.

*Mapkapk3* can be activated by three different mitogen-activated-protein-kinases (MAPK): p38, extracellular-signal-regulated kinase (ERK) and Jun-N-terminal kinase (JNK) (Ludwig et al., 1996; Zakowski et al., 2004). These three MAPK pathways can all become activated by oncogenic RAS, although ERK and JNK are generally more strongly activated than p38 (Chen et al., 2000). The ERK pathway is well known for its stimulatory effect on cell cycle progression, however, high levels of activated ERK can induce cell cycle arrest. This controversy is illustrated by ERK’s ability to induce both Cyclin D1 and p21<sup>CIP1</sup> (Sebolt-Leopold and Herrera, 2004; Roovers and Assoian, 2000). The stress-activated p38 pathway functions in inhibiting proliferation and counteracting transformation (Loesch and Chen, 2008; Han and Sun, 2007). Of relevance, various ways of cross talk have been reported between the different MAPK pathways. Particularly, active MEK could induce p38 and additionally, activation of p38 by oncogenic RAS required the RAF/MEK/ERK pathway (Han and Sun, 2007; Wang et al., 2002; Chen et al., 2000). On the other hand, activated p38 could inhibit MEK/ERK (Aguirre-Ghiso et al., 2003; Li et al., 2003) and has also been reported to inhibit the proliferation-stimulating JNK pathway (Loesch and Chen, 2008; Chen et al., 2000).

Given the reported growth inhibitory effect of the p38 pathway, we wondered whether down-regulation of p38 could promote transformation of RAS<sup>V12</sup>/Rb<sup>-/-</sup>p107<sup>-/-</sup> MEFs. Indeed, treatment with the p38 inhibitor SB203580 supported anchorage-independent growth of these cells (Fig. 2B). A tumor suppressor role for p38 could simply be explained by the aforementioned inhibition of the proliferation- and survival-stimulating ERK pathway (Sebolt-Leopold and Herrera, 2004; Li et al., 2003). Along these lines, Aguirre-Ghiso and co-workers (2003) suggested that a high p38/ERK ratio correlated with cell cycle arrest in cancer cell lines. Additionally, experiments performed by Wang and colleagues (2002) pointed to a role for p38 in cell cycle arrest: chemical inhibition of p38 or chemical inhibition of MEK, which in turn inhibited p38, counteracted RAS<sup>V12</sup>-induced senescence. Thus, their results suggest that RAS<sup>V12</sup>-induced senescence is mediated via MEK/p38.

In addition to inhibiting MEK/ERK, p38 can induce several cell cycle inhibitors and tumor suppressors, including p19<sup>ARF</sup>, p53, p21<sup>CIP1</sup>, p27<sup>KIP1</sup> and p16<sup>INK4A</sup> (Han and Sun, 2007), which can all contribute to growth inhibition. In line with this, Bulavin and co-workers (2004) showed that ablation of the WIP1 phosphatase in MEFs raised the level of activated p38, which correlated with inhibition of tumor formation in nude mice via the p16<sup>INK4A</sup>/p19<sup>ARF</sup> pathways, however, this effect was independent of p53. In contrast, Dolado and co-workers (2007) suggested that p38 could inhibit RAS<sup>V12</sup>-induced transformation independently of p16<sup>INK4A</sup>/p19<sup>ARF</sup> and also independently of affecting ERK. In fact, they
correlated p38 with the induction of apoptosis in response to reactive oxygen species (ROS), which were produced in response to RAS\textsuperscript{V12} signaling. Similarly, Nicke and co-workers (2005) suggested that ROS, induced by RAS\textsuperscript{V12}, caused activation of p38. In conclusion, several different mechanisms could contribute to the tumor suppressor role of p38 in our system.

We consider it likely that p38 suppresses transformation via the induction of cell cycle inhibitors, since (1) our previous studies clearly demonstrated that downregulation of the p19\textsuperscript{ARF}/p53/p21\textsuperscript{CIP1} pathway induced transformation of RAS\textsuperscript{V12}/pocket protein-deficient MEFs and (2) Mapkapk3 is possibly involved in regulation of p19\textsuperscript{ARF}. Experiments by Voncken et al. (2005) showed that Mapkapk3 can phosphorylate the Polycomb group protein BMI1, resulting in its release from the chromatin. BMI1 is part of the Polycomb-Repressive Complex which represses the INK4A/ARF locus (Bracken et al., 2007; Jacobs et al., 1999). Together, this suggests that Mapakpk3 might inhibit transformation via phosphorylation and displacement of BMI1 upon loss of anchorage, resulting in the induction of p19\textsuperscript{ARF}. Future studies are required to address this possibility.

Whereas Mapkapk3 is relatively uncharacterized, Mapkapk2 is a well established target of the p38 pathway. A growth suppressive role for p38/Mapkapk2 was demonstrated by the observation that expression of p38 or of Mapkapk2 inhibited RAS\textsuperscript{V12}-induced S-phase entry in serum-deprived NIH3T3 cells. Strikingly, both Mapkapk2 and p38 could inhibit RAS-induced transcription in \textit{in vitro} reporter assays (Chen et al., 2000). Additionally p38/Mapkapk2 was implicated in DNA-damage-induced cell cycle arrest in the absence of p53 (Reinhardt et al., 2007; Manke et al., 2005). We did not detect transcriptional induction of \textit{Mapkapk2} upon loss of anchorage, in contrast to \textit{Mapkapk3}, which was clearly induced. However, since Mapkapk proteins are activated by phosphorylation, the possibility remains that Mapkapk2 is activated upon loss of anchorage and performs a similar role as Mapkapk3.

In conclusion, we identified downregulation of Mapkapk3 or p38 as an additional event that could promote anchorage-independent growth of RAS\textsuperscript{V12}-expressing, pocket-protein-deficient MEFs. These results point to a tumor suppressor role for Mapkapk3 signaling upon loss of anchorage. Future experiments are required to determine the upstream and downstream mechanisms that mediate this growth-inhibitory role, and whether a similar role can be performed by the related Mapkapk2.
Materials and Methods

Cell culture
MEFs were cultured in GMEM (Invitrogen/Gibco), containing 10% fetal calf serum, 1 mM nonessential amino acids (Invitrogen/Gibco), 1 mM sodium pyruvate (Invitrogen/Gibco), 100 units/ml penicillin (Invitrogen/Gibco), 100 μg/ml streptomycin (Invitrogen/Gibco) and 0.1 mM β-mercaptoethanol and incubated at 37 °C in the presence of 5% CO₂.

Retroviral supernatants were produced by calcium phosphate transfection (Invitrogen) of phoenix cells with 16 μg of the desired construct and 4 μg pCL-Eco. Forty-eight h post transfection, retroviral supernatant was filtered using 0.45 μm filters (MCE membrane, Millipore) and either used directly or immediately frozen using a dry-ice ethanol bath and stored at -80 °C. After harvesting viral supernatant, phoenix cells were supplemented with GMEM containing media supplements as described above, and supernatant was again harvested using the same procedure, with an interval of at least 6 h. Subconfluent cell cultures were infected twice with retroviral supernatants, supplemented with polybrene to a concentration of 4 μg/ml during a time-span of at least 6 h per infection. For serial infections, MEFs were cultured in non-virus containing media for at least 36 h between infections and reseeded before infection to obtain optimal cell density.

Culturing without anchorage was performed in either soft agar or in methylcellulose-containing medium, as described in Vormer et al. (2008). In short, MEFs were suspended in a 37 °C, 0.35% soft agar solution (low gelling agarose type VII from Sigma) in GMEM containing 10% fetal calf serum, the same medium supplements as mentioned above plus gentamicin to a concentration of 0.02 mg/ml (Invitrogen/Gibco) and plated on top of a pre-casted 1% soft agar layer. For methylcellulose assays, MEFs were suspended in a 37 °C, 1.3% methylcellulose solution (diluted from 2.6% methylcellulose medium, Stem Cell Technologies, catalog no. H4100) in GMEM supplemented with fetal calf serum to a concentration of 10%, penicillin to a concentration of 100 units/ml (Invitrogen/Gibco), streptomycin to a concentration of 100 μg/ml (Invitrogen/Gibco) and gentamicin to a concentration of 0.02 mg/ml (Invitrogen/Gibco) and plated in ultra-low-attachment surface plates (catalog no. 3471; Corning incorporated). Pictures were taken using a non-phase-contrast lens (2.5x magnification) and assembled using ‘Axiovision 4.5’. Cells were harvested by suspending 4 ml of methylcellulose culture with 40 ml ice-cold phosphate-buffered saline (PBS) (Invitrogen/Gibco), followed by centrifugation and aspiration of methylcellulose-PBS.

Constructs and PCR analysis
The ERM vector was kindly provided by Dr. Z. Songyang, pBABE-RASV12 by Dr. T Brummelkamp and pCL-Eco by Dr. D. Peep. The pEYK-TBX2 vector was previously isolated from MEFs infected with the pEYK-MCF7 library (Vormer et al., 2008), which was a gift of Dr. G.Q. Daley.

PCR analysis (Fig. 1B) which demonstrated the ERM-Mapkapk3 fusion in a soft agar colony isolated from the insertional mutagenesis screen was performed with the following primers: ERM forward (fv): 5’ GGC CAT GGA CAC CTA CCG CTA CAT CG 3’; Intron 2 fv: 5’ CTC ATA AGC TGA CCC ACC CT 3’; Intron 2 reverse (rv): 5’ CCT TGA CTC ACC TTC ATG ATC C 3’.

For analysis of the ERM-Mapkapk3 fusion at the mRNA level (Fig. 1C), reverse transcription was performed using a primer annealing to the 3’ end of Mapkapk3, downstream of the stopcodon (5’ CCC CAA GTT CAA TGT GAC AC 3’). The RT fragment was subsequently amplified using the same primer and a primer annealing to the myristylation signal of ERM (5’ ACC ATG GGG AGC AGC AAG AGC AAA CCA AAA GAC CCC AGC CAA CGC 3’ (Liu et al., 2000)). The resulting RT-PCR product was cloned into pGEM-Teasy and sequenced using primers annealing to the T7 or Sp6 sequence present in pGEM-Teasy. Next, the ERM-Mapkapk3 RT-PCR product was cloned from
Mapkapk3 suppresses anchorage-independent growth

pGEM-Teasy into pBABE-bleomycin using the EcoRI site, generating pBABE-ERM-Mapkapk3.

Full-length, wild-type Mapkapk3 was reverse transcribed using the primer annealing to the 3’ end of Mapkapk3 (see above), amplified using this primer and a primer located upstream of the Mapkapk3 start codon (5’ GCT GTA CGT GCC TCT GGA C 3’), cloned into pGEM-Teasy and subsequently into pBABE-bleomycin using the EcoRI site, generating pBABE-Mapkapk3.

Targeting sequences in pRetroSuper-Mapkapk3 vectors are: GCT CCT CAG CCT CAC AAG G (pRS-Mapkapk3-1) or GGA AAA AGC AGG CAG GCA GC (pRS-Mapkapk3-2).

Insertional mutagenesis screen

Rb⁻/⁻p107⁻/⁻p130⁻ MEFs were retrovirally infected with pBABE-RASV12 and ERM and subsequently cultured in 0.35% soft agar. Soft agar colonies were isolated using sterilized glass pipettes and propagated under adherent conditions, after which genomic DNA (gDNA) was isolated by lysis in 0.1 M TrisHCl pH 8.5, 5 mM EDTA, 0.2 M NaCl, 0.2 % SDS and 100 µg/ml Proteinase K (O/N at 55 ºC) and subsequent ethanol precipitation. gDNA was dissolved in Milli-Q water and purified using phenol/chloroform extraction. The position of the ERM integrations in the genomic DNA was determined using splinkerette PCR conform Mikkers et al. (2002), followed by sequencing.

Micro-array analysis

RNA was isolated using the RNeasy mini kit (Qiagen, catalog nr 74106). mRNA amplification using the Superscript RNA Amplification System (Invitrogen, catalog no. L1016-01), labeling and hybridization to MouseWG-6 v2.0 Expression BeadChip (Illumina) was performed as described on: http://microarray.nki.nl/download/protocols.html.

Acknowledgements

We thank Ron Kerkhoven and the members of the central microarray facility of the Netherlands Cancer Institute for microarray analysis and advice. This work was financially supported by the Dutch Cancer Society, grant nr NKI 2002-2634.

References


Chapter 3


Nicke, B., Bastien, J., Khanna, S.J., Warne, P.H., Cowling, V., Cook, S.J., Peters, G., Delpuech, O.,
Mapkapk3 suppresses anchorage-independent growth


