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FoxK2 is Required for Cellular Proliferation and Survival

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FoxK2 is a forkhead transcription factor expressed ubiquitously in the developing murine central nervous system. Here we investigated the role of FoxK2 in vitro and focused on proliferation and cellular survival. Knockdown of FoxK2 results in a decrease in BrdU incorporation and H3 phosphorylation, suggesting attenuation of proliferation. In the absence of growth factors, FoxK2 knockdown results in a dramatic increase in caspase 3 activity and propidium iodide positive cells, indicative of cell death. Additionally, knockdown of FoxK2 results in an increase in the mRNA of Gadd45α, Gadd45γ, as well as an increase in the phosphorylation of the mTOR dependent kinase p70S6K. Rapamycin treatment completely blocked the increase in p70S6K and synergistically potentiated the decrease in H3 phosphorylation upon FoxK2 knockdown. To gain more insight into the proapoptotic effects upon FoxK2 knockdown we screened for changes in cellular viability (Chipuk et al., 2010). When stress levels outbalance metabolic needs, a major signaling pathway that translates nutrient availability into proliferation and survival is the mTOR pathway (Sengupta et al., 2010). mTOR integrates signals from amino acids, nutrient, AMP levels, and AKT activity to control the activity of a variety of downstream targets which include p70S6K and 4E-BP1. Both p70S6K and 4E-BP1 regulate protein translation, proliferation, and cellular metabolism (Sengupta et al., 2010). Upon loss of metabolic input the cell may undergo mitochondrial BCL2 dependent apoptosis. BCL2 proteins can be divided into pro-apoptotic and anti-apoptotic members. The pro-apoptotic and anti-apoptotic BCL2 members can often interact and cancel out each-others function, thus their mutual balance is of extreme importance for cellular viability (Chipuk et al., 2010). When stress levels exceed metabolic thresholds the balance between them can shift towards pro-apoptotic BCL2 members (Chipuk et al., 2010).

Here we characterize the effects of FoxK2 knockdown using an in vitro loss of function approach. We confirm that FoxK2 is indeed required for proliferation as has been described, additionally we have linked the mTOR pathway to FoxK2 as loss of FoxK2 increased p70S6K activity. Combinatorial knockdown of FoxK2 and mTOR inhibition with rapamycin potentiates the effects on inhibition of cellular proliferation, suggesting mTOR compensates partly for FoxK2 loss. Since it has been reported that a mutant FoxK2 can not be tolerated by the cell and induces caspase 3 activity (Marais et al., 2010), we screened Bcl2 genes after FoxK2 knockdown and identified Puma and Noxa as genes that were increased upon FoxK2 loss. Overall our study identifies the mTOR pathway and the Bcl2 dependent apoptotic pathway as important interactors of FoxK2 function.

Conflict of interest: The authors have declared that no competing interests exist.

Lars P. van der Heide and Patrick J.E.C. Wijchers contributed equally to the work and should both be considered first author.

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Materials and Methods

Isolation of the mouse FoxK2 cDNA

From adult mouse brain tissue (C57/B16), total RNA was isolated using TRIzol Reagent (Invitrogen) and reverse transcribed using SuperScript II (Invitrogen) in combination with both oligo (dT) and random hexamer primers. A 169 bp fragment was isolated in a PCR (annealing temperature of 55 °C) using degenerate oligonucleotides 5'-AGGCCACSTACWCTATG-3' (forward) and 5'-CACAACCTCTYCTKAAM-3' (reverse), deduced from a subset of forkhead transcription factors. Multiple expressed sequence tags (ESTs) are available (e.g. Genbank accession 0-AAGCCACSSTACWCTATG-3').

Cells of all used cell types were cultured in Dulbecco's modified Eagles medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (HIFCS), 100 units/ml penicillin, 100 units/ml streptomycin, and 2 mM L-glutamine in a humidi 00% CO2 at 37 °C. Twenty-four hours before transfection, cells were serum starved for 24 h before treatment with TGFβ (1 ng/ml, kindly provided by Prof. Peter ten Dijke).

FoxK2-EGFP intracellular localization

The full length FoxK2 coding sequence was fused in frame to an enhanced green fluorescent protein (GFP) reporter gene. Using primers containing appropriate restriction sites, the FoxK2 coding sequence (without the STOP codon) was positioned upstream of the pEGFP-N1 vector (Clontech), whereby the resulting construct (FoxK2-EGFP) codes for a fusion protein of FoxK2 linked to GFP, which contains the STOP codon. HEK-293 cells were grown on coverslips, and cultured for another 24 h after calcium phosphate transfection with expression vectors in medium with or without 10% (v/v) HIFCS. Cells were fixed in 4% PFA, embedded in Dabco-mowiol and analyzed by fluorescence-microscopy.

RT-PCR screen

Randomly selected mouse and human cell lines were cultured in 100 mm dishes, and total RNA was extracted using TRIzol Reagent (Invitrogen), and subsequently reverse transcribed using Superscript II (Invitrogen) in combination with both oligo (dT) and random hexamer primers. Using intron-spanning FoxK2 primers in exons 5 and 6 (mouse forward 5'-ATTCAATTCGCCACCATCTCCT-3' and reverse 5'-AAACCTGGCCCTCCTGATG-3'; human forward 5'-GCCCAATCTCCTCTGAACT-3' and reverse 5'-TTC-TGGATGACAGCAG-3', annealing temperature of 56 °C for both primer sets), we were able to distinguish between PCR end-products from cDNA (353 bp and 335 bp for mouse and human respectively) and genomic origin (1,044 bp and 1,413 bp).

siRNA design and efficiency

To study FoxK2 function in vitro, we used synthetic siRNAs to knock down FoxK2 expression. Four FoxK2 specific siRNA duplexes were purchased (Proligo), 5'-UUACUGUCACCGGC-CAGCUTT-3' (sense strand, si1280), 5'-GCCCAACAUUGGAGAG-GAGACTT-3' (si1766), 5'-UUUUAACGGCAUUUGU-ATT-3' (si886) and 5'-UUUGAGAAUACAGGUGUUCCTT-3' (si909). The numbers in the siRNA names refer to the relative position in the FoxK2 coding sequence. As controls, a scrambled siRNA was designed with a similar nucleotide composition as si1280 (siCON, 5'-GGGAGCUGUUCACACAGUCAGCT-3') and a functional GFP-specific siRNA (siGFP) was purchased from Dharmaco (GFP Duplex III). We never observed any effects using siCON compared with pBluescript (pSK) carrier DNA controls.

Cell lysis and Western blot

For GFP westerns cells were lysed with 50 mM TRIS, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 100 mM sodium fluoride and 1 mM sodium vanadate on ice. Insoluble material was removed from the sample by brief centrifugation. Concentrated SDS sample buffer containing 66 mM TrisHCl pH 6.8, 3% (w/v) SDS, 5% (v/v) glycerol, 0.001% (w/v) bromophenol blue, and 2% (v/v) beta-mercaptoethanol was added to the samples before samples were heated for 5 min at 100 °C. Protein samples were separated by 9% SDS-PAGE. After electrophoresis protein was transferred to nitrocellulose membranes (Amersham) using a Biorad wet Blotting apparatus according to manufacturer’s instructions. Protein transfer and blotting efficiency were verified with ponceau-S. Blots were blocked for 1 h at room temperature in PBS containing 0.05% Tween-20 (PBS-Tween) and 5% milk powder. Membranes were incubated with anti-GFP, diluted 1:10,000 in PBS-Tween, for 1 h at room temperature. Secondary anti-rabbit antibody-HRP conjugate was diluted 1:50,000 in PBS-Tween and incubated for 45 before visualization with ECL detection substrate (Amersham) and HyperFilm (Amersham). For all other westerns, cells were grown as described and after treatment lysed directly in sample buffer. Samples were sonicated 90 s using a bioruptor (Diagenode) and heated for 5 min at 95 °C. Cleaved caspase 3 (CST), phospho-Smad2 (CST), Smad2/3 (BD), were diluted 1/1000 in TBS-T and heated for 5 min at 95 °C. Cleaved caspase 3 (CST), phospho-Smad2 (CST), Smad2/3 (BD), were diluted 1/1000 in TBS-T and incubated overnight at 4 °C. ECL detection of proteins was performed using a LICOR detection device according to manufacturer’s instructions. Antibodies to p70S6K, AKT, phospho-AKT as well as rapamycin were a kind gift of Dr. H. Krugers.

Immunocytochemistry

To test the efficiency of the siRNAs in murine cell lines, NIH3T3 and ActT20 cells were grown on coverslips in 6-well plates and transfected with 0.6 µg siRNA or pSK and 0.6 µg FoxK2-EGFP vector using Lipofectin (Invitrogen). Cells were subsequently grown for 48 h in the presence of serum, fixed in 4% PFA and embedded in Dabco-mowiol. Using fluorescence microscopy, the number of green fluorescent cells was counted for each coverslip.

BrdU immune-cytochemistry

NIH3T3 cells were seeded on coverslips in 12-wells plates in medium containing 10% (v/v) HIFCS. After 24 h, cells were pulse-labeled with 0.3 µg siRNA or pSK and 0.6 µg FoxK2-EGFP vector using Lipofectin (Invitrogen) according to manufacturer’s instructions. After 48 h, cells were pulse-labeled for 1 h with 10 µM BrdU (Upstate) in serum.
supplemented medium at 37 °C. Cells were fixed for 20 min in 4% PFA, and subsequently permeabilized with 0.2% Triton-X-100 in PBS (PBST) for 10 min. Following blocking for 20 min with 10% HIFCS in PBS, cells were treated for 90 min with DNAseI (50 U/ml) in PBS containing 0.5 mM CaCl2 and 0.9 mM MgCl2. Cells were then incubated for 1 h with anti-brdU (1:200, Neomarkers) in PBST in the dark, followed by 1 h with Cy3 conjugated donkey anti-rabbit IgG (1:400, Jackson ImmunoResearch Laboratories, Inc.) in PBST in the dark. Finally coverslips were embedded in Dabco-mowiol and analyzed by fluorescence microscopy. All steps were performed at room temperature unless stated otherwise. For 100 green fluorescent cells, BrdU incorporation was evaluated as either positive or negative without prior knowledge of sample identity. Experiments were performed at least four times.

Phospho-Histone H3 (Ser10) immuno-cytochemistry

NIH3T3 cell culture and transfection were essentially similar to the BrdU stained cells. Control transfections with pSK were not considered a requirement anymore since we have never observed any difference between siCON and pSK. After 48 h, cells were fixed for 20’ in 4% PFA and permeabilized in PBST for 3’. Cells were blocked for 30’ with 5% HIFCS in PBS and incubated for 2 h with -phospho-Histone H3 (Ser10, Upstate) diluted 1:200 in PBS in the dark. After 1 h incubation in the dark with Cy3 conjugated goat anti-rabbit IgG (1:400, Jackson ImmunoResearch Laboratories, Inc.) coverslips were embedded in Dabco-mowiol and analyzed by fluorescence microscopy. All steps were performed at room temperature unless stated otherwise. For 100 green fluorescent cells, phosphorylation of histone H3 was evaluated as either positive or negative without prior knowledge of sample identity. Experiments were repeated at least four times.

Propidium iodide labeling

NMuMG cells were transfected with FoxK2 siRNA as described previously and grown grown for 48 h in serum containing medium. After overnight serum starvation PI was added directly to the growth medium (1 mg/ml) and visualized with a Leica microscope.

Flow cytometry

NIH3T3 cells were grown in 100 mm dishes and transfected and treated as described above, with the amounts adjusted to the number of cells. For PI analysis, pEGFP-n1 was substituted by a constitutive nuclear localization which is not in genomic contamination, we employed RT-PCR and RT-QPCR to screen several randomly selected mouse and human cell lines for FoxK2 expression. Consistent with the in vivo expression in proliferating tissues, FoxK2 transcripts were detected in all cell lines examined in this study (NIH3T3, NMuMG, N2a, Att20, HEK293, (data not shown)). The activity of several forkhead factors is regulated post-translationally by growth factors whereby phosphorylation of forkhead proteins results in intracellular translocation from the nucleus to the cytoplasm (Van Der Heide, 2004) (Wolfur et al., 2003). Therefore, to visualize the intracellular localization of FoxK2, we transfected cells with an expression vector coding for FoxK2 fused to Enhanced Green Fluorescent Protein (EGFP), in serum-rich (growth factor containing) or serum-free medium. Under normal, serum-rich conditions, FoxO1 mostly resides in the cytosol and is translocated to the nucleus upon serum-starvation, where it can regulate the expression of downstream target genes (Fig. 1A) (Jacobs et al., 2003; Van Der Heide, 2004). In contrast to FoxO1, the intracellular location of FoxK2 was exclusively nuclear, both in the presence and absence of serum (Fig. 1A). EGFP alone showed no preference for nucleus or cytosol. Thus, FoxK2 has a constitutive nuclear localization which is not influenced by signaling pathways induced by serum.

Validation of FoxK2 siRNA

To study FoxK2 function in more detail, we first analyzed several synthetic siRNA duplexes for their efficiency to knock down FoxK2 levels. We therefore co-transfected HEK-293 cells with siRNAs against the murine FoxK2 gene together with a FoxK2-EGFP expression vector, and carefully measured the efficiency by analyzing the levels of FoxK2-EGFP fusion protein by Western blot. Using an antibody against GFP, we observed the expected 100 kDa size fusion protein (25 kDa GFP and 75 kDa FoxK2). Of the four siRNAs examined, si886 (referring to the basepair position in the coding sequence) and si909 appeared highly efficient in down-regulating FoxK2-EGFP levels, whereas si1280 and si766 had only minor effects compared to control siRNA (Fig. 1B). As expected, FoxK2-specific siRNAs had no effect on GFP levels alone, and only the GFP-specific siRNA reduced both the FoxK2-EGFP fusion protein and GFP alone. To confirm the efficiency of si886 and si909 in mouse cell lines and by another analysis method, we transfected NIH3T3 cells with FoxK2-EGFP in combination with the siRNAs. To analyze the level of FoxK2-EGFP fusion protein
we counted the total number of transfected cells on small coverslips. In both cell lines, si886, si909, and siGFP caused a reduction of approximately 90% in the number of GFP positive cells, compared to control siRNA. Replacing siRNA with carrier DNA (pBluescript SK (pSK)) resulted in similar numbers as control siRNA, eliminating the possibility of an a-specific effect of siRNA (data not shown). From these experiments we chose si886 and si909 for further use in functional experiments.

FoxK2 deficiency leads to reduced proliferation in vitro

To determine whether changes in FoxK2 levels can influence the cell cycle, we labeled transfected cells for either BrdU incorporation or phosphorylated histone H3 (at Serine 10), markers for S- and M-phase, respectively. We transfected NIH3T3 fibroblasts with a FoxK2 siRNA in combination with a vector for EGFP to select for transfected cells, and determined for each green fluorescent cell whether it was positive or negative for these markers. Both control samples (siCON and pSK) gave similar results in the BrdU experiments, and as differences have never been observed, siCON has been used as control in subsequent experiments. With si886, we observed a decrease both in the number of BrdU positive cells (Fig. 2A, \( P < 0.05 \)) and in cells labeled for phosphorylated histone H3 (Fig. 2B, \( P < 0.05 \)). Similar results were obtained for si909, although the decreases were smaller and non-significant.

Together, the reduction of cells in S- and M-phase suggests that FoxK2 deficiency results in a reduced number of proliferating cells. Several forkhead factors are known for their role in regulating cell cycle entry and/or transition into G0 phase. However, the yeast homologue of FoxK2 (Fkh2) is a key factor in transcriptional regulation of the G2/M phase of the cell cycle in Saccharomyces cerevisiae (Wittenberg and Reed, 2005), suggesting that the proliferation defects observed in the absence of FoxK2 could be due to an impairment in progression from G2 to M phase of the cell cycle. We therefore analyzed the cell cycle distribution by measuring DNA content in cells co-transfected with a membrane localized spectrin-GFP (Kalejta et al., 1997) and different FoxK2 siRNAs, using Propidium Iodide (PI). Strikingly, we did not find any apparent changes in the cell cycle distribution when transfected with siRNAs against FoxK2, with a similar percentage of cells present in all phases of the cell cycle compared to control siRNA (66% G1/G0, 17% G2/M, 17% S) (Fig. 2C). This suggests that the decreased proliferation is not due to a change or defect in the cell cycle machinery. Thus, in contrast to Fkh2 in yeast, FoxK2 does not regulate G2/M progression in this experimental setup.

FoxK2 knockdown increases p70S6K phosphorylation

FoxK2 reduced the amount of phospho-histone 3 positive cells (Fig. 2B), to confirm this finding in another cell type we examined the effects of FoxK2 knockdown in the mouse...
In NMuMG cells FoxK2 knockdown resulted in a reduction of histone 3 phosphorylation as detected with Western-blotting (Fig. 3A). As a positive control for the assay we conducted the same experiment in the absence of serum, forcing cells in growth arrest. Serum withdrawal lead to a substantial decrease in histone 3 phosphorylation in the control, which was reduced even further upon FoxK2 knockdown. Since the withdrawal of serum reduced histone 3 phosphorylation we speculated that adding fresh serum containing medium might rescue the effects of FoxK2 knockdown. Replacing the growth medium 24 h before analysis of histone 3 phosphorylation resulted in a dramatic increase in phosphorylation as compared to the replacing of the growth medium 48 h before the analysis (data not shown). Upon FoxK2 knockdown a reduction in histone 3 phosphorylation can be observed when the serum containing medium is replaced 48 h before the measurement but not when replaced 24 h before the measurement. Possibly, the effects of FoxK2 become apparent when the growth factors in the growth medium are more depleted. Since the presence of serum effected the level of histone 3 phosphorylation as does FoxK2 knockdown, we speculated that FoxK2 might have effects on pathways sensitive to nutrient availability, such as the mTOR pathway. Therefore, we examined the level of p70S6K phosphorylation as this kinase is controlled directly by mTORC1 (Sengupta et al., 2010). Counter intuitively FoxK2 knockdown resulted in an increase in p70S6K phosphorylation (Fig. 3B), which suggests a reduction in mTOR activity is not the cause of decreased histone 3 phosphorylation and BrdU incorporation.

Fig. 2. Effect of FoxK2 knock-down on proliferation in NIH3T3 cells. A: Representation of microscopic view of GFP positive cells (left column), red fluorescent labeled BrdU molecules (S-phase marker, middle column) and both fluorescent channels at the same time (merged, right column). The histogram displays the average percentage of BrdU-positive cells for transfected populations. Error bars indicate standard error of the mean. B: As in (A), but for phosphorylated histone H3 (M-phase marker). C: Cell cycle distribution of cells transfected with different siRNAs. Cells were co-transfected with spectrin-GFP and stained with PI. D: Histogram displays the percentages of subdiploid (DNA content < 2n) cells in histograms in (C).
mTOR partly compensates for the loss of FoxK2

Since FoxK2 knockdown resulted in a reduction in proliferation we examined the transcripts for Growth Arrest and DNA-Damage 45α, β, and γ (Gadd45α, Gadd45β, and Gadd45γ) as possible mediators of growth arrest. Of these three Gadd45 genes, the Gadd45α and Gadd45γ were upregulated in response to FoxK2 knockdown in NMuMG cells (Fig. 4A). Next we treated the cells with rapamycin to inhibit mTOR and explore a potential link between mTOR and Gadd45 upregulation. Rapamycin slightly diminished the expression of both transcripts, suggesting they are indeed sensitive to mTOR activity (Fig. 4B). To further investigate the link between mTOR mediated activation of p70S6Kinase after FoxK2 knockdown we treated cells with rapamycin and analyzed the phosphorylation state of histone 3 24 h later. Rapamycin treatment effectively blocked all p70S6K phosphorylation but did not have a major effect on control cells, whereas it greatly potentiated the loss of H3 phosphorylation upon FoxK2 knockdown (Fig. 4C). No clear effect of rapamycin and FoxK2 can be observed in the control condition, because fresh serum containing medium was added 24 h before the measurement.

FoxK2 influences cell survival

Interestingly, in the DNA content histograms of NIH3T3 cells, as shown in Figure 2C, we observed an increase in the number of cells with a DNA content of less than 2n upon FoxK2 knockdown, a feature indicative of cell death (14). To examine the possibility that in response to FoxK2 knockdown cells undergo cell death we screened for a number of Bcl2 genes (Fig. 5A). The genes that showed at least a 50% change were subjected to a full n = 4 analysis. In this screen we identified Bbc3 (Puma), Bcl2l2, Bak, Bok, and Noxa as changed (Fig. 5A). After the full n = 4 analysis only Bok was found not to be significantly changed (Fig. 5B). To again test if the change observed could be mediated by mTOR activity we treated the cells with rapamycin and analyzed the two Bcl2 members with the largest fold change upon FoxK2 knockdown. Puma expression was not affected by rapamycin treatment, whereas Noxa was slightly upregulated (Fig. 5C), suggesting mTOR activity slightly reduces the expression of Noxa. To further study the role of FoxK2 in cellular survival we investigated the effect of FoxK2 knockdown in NMuMG cells. Knockdown of FoxK2 with si886 or si909 lead to increased cleavage of caspase 3 (Fig. 6A,B), a process heavily involved in the execution of cell death. To confirm that the increase in cleaved caspase 3 was indicative of cell death, we measured the amount of PI positive cells after FoxK2 knockdown. Indeed, knockdown of FoxK2 led to a strong increase in the amount of PI positive cells, indicating membrane permeability and the occurrence of cell death (Figs. 6D and E). Since FoxK2 knockdown increased the basal level of cell death we speculated that knockdown of FoxK2 would also reduce the cellular resistance to apoptotic stimuli. To this end,
we treated NMuMG cells with TGFβ, a well described inducer of apoptosis in this cell type (Van der Heide, 2011). TGFβ induced an increase in cleaved caspase 3 as expected, which was even more pronounced in combination with FoxK2 knockdown (Figs. 7A and B). Since TGFβ potentiates the amount of caspase 3 cleavage in the FoxK2 knockdown condition, we tested with PI if the increase in caspase 3 activity correlated with cell death. Under basal conditions few PI positive cells were present and greatly increased in number after FoxK2 knockdown. TGFβ increased the amount of PI positive cells to greater extent than FoxK2 knockdown, but the combination of TGFβ and FoxK2 knockdown lead to the highest abundance of PI positive cells (Fig. 7C). Overall FoxK2 knockdown decreases proliferation and induced cell death, presumably the mTORC1 is activated as a compensatory mechanism.

**Discussion**

In our study we show that FoxK2 is required for cellular proliferation and survival. Knockdown of FoxK2 clearly reduced
H3 phosphorylation and BrdU incorporation (Fig. 2–4), whereas it induced caspase 3 cleavage and cell death (Fig. 6–7). The mechanism underlying FoxK2 function towards cell proliferation and survival is at present unclear. Since FoxK2 is a transcription factor, it is likely to regulate an anti-apoptotic or survival gene. Genes we identify to be involved in FoxK2 metabolism are members of the Gadd45 (Fig. 4) and Bcl2 family (Fig. 5).

A recent study hinted towards a role for FoxK2 in cell-cycle as it was shown that FoxK2 is phosphorylated by a CDK1-Cyclin B1 complex in a cell cycle dependent manner (Marais et al., 2010). Interestingly, when the FoxK2 phosphorylation sites were mutated to mimic a non-phosphorylated state the cells died, the authors suggested that this form of FoxK2 cannot be tolerated by the cell (Marais et al., 2010). The target genes regulated by FoxK2 were not identified. A follow-up study by the same group showed that FoxK2 functions to promote AP1 dependent regulation of target genes (Ji et al., 2012). However, in this second study no specific link between FoxK2, AP1, and apoptosis is reported. Here we clearly show that specific BCL2 target genes are up-regulated in response to FoxK2 knockdown.

An additional mechanism that may explain how the loss of FoxK2 results in an inhibition of proliferation and potentiation of cell death was described recently in a study that suggested a role for FoxK2 in G/T DNA mismatch repair (Fujii and Nakamura, 2010). Interestingly, FoxK2 binds with higher affinity to G/T mismatch DNA than to the FoxK2 consensus motif. Since proliferating cells are more sensitive to DNA damage, they require a highly efficient DNA-repair machinery to prevent cumulative damage and DNA mutations. Possibly, proliferating cells utilize the G/T mismatch repair capacity of FoxK2 to repair DNA damage and prevent accumulation of DNA damage over time. Conversely, loss of FoxK2 would lead to inefficient DNA repair, accumulation of DNA damage and ultimately cell death.

**FoxK2 regulates p70S6K**

Completely novel is our finding that knockdown of FoxK2 induces the phosphorylation of p70S6K (Fig. 3). Since p70S6K

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**Fig. 5.** Multiple Bcl2 genes are regulated after FoxK2 knockdown in NMuMG cells. A: single point samples were screened for changes in expression after RNA isolation and RT-qPCR as described above. Changes more than 50% were reanalyzed using a complete set of 4 individual samples n = 4 (B). Significance was determined with a student’s t-test P < 0.05. C: Cells were treated with rapamycin to monitor its effects on Puma and Noxa expression after RT-QPCR. Significance was determined with a student’s t-test P < 0.05.
phosphorylation could be completely blocked with rapamycin it is highly likely that the increase in p70S6K reflects an increased activity of mTORC1 and possibly the upstream mTORC1 kinase AKT (Fig. 4). AKT is a kinase which can positively influence cellular survival by phosphorylating and inactivating pro-apoptotic proteins such as the BCL2 protein Bad and the FoxO transcription factors (Van Der Heide et al., 2004; Chipuk et al., 2010). The mechanism leading to AKT/p70S6K activation after FoxK2 knockdown is unclear at present. mTORC1 integrates various metabolic cues and regulates survival, proliferation, and protein translation (Sengupta et al., 2010). Since FoxK2 knockdown reduces proliferation and induces cell death, it is likely mTORC1 activity represents a compensatory mechanism. This is further supported by the fact that upon rapamycin treatment histone 3 phosphorylation is greatly diminished upon FoxK2 knockdown. Possibly Gadd45α and Gadd45γ are part of the compensatory mechanism induced by mTORC1 as they are both sensitive to rapamycin treatment and upregulated after FoxK2 knockdown. GADD45 proteins have multiple functions which include involvement in cell cycle arrest, DNA repair, cell survival and apoptosis in response to environmental and physiological stress, as well as a role in development and carcinogenesis (Liebermann et al., 2011). The biological outcome after

**Fig. 6.** FoxK2 knockdown leads to increased cell death. A: NMuMG cells were transfected with FoxK2 siRNA (si886 and si 909) or control siRNA. Protein cell lysates were analyzed for content of beta actin or cleaved caspase 3. B: Densitometric quantification of (A) using ImageJ. Level of Caspase 3 was corrected for beta actin content. C: QPCR analysis of FoxK2 transcript level after FoxK2 knockdown with siRNA. FoxK2 levels were corrected for TBP content. Experiment was performed in triplicate. D: Cells transfected with FoxK2 siRNA (siRNA 886) were treated with propidium iodide (PI) to visualize dying cells. E: Quantification of PI positive cells after removal of floating cells with a PBS wash.
GADD45 expression depends on the magnitude and type of stress stimulus and cell type. Interestingly, GADD45 can interact with PCNA to promote nucleotide excision repair of DNA, linking FoxK2 and GADD45s to G/T mismatch repair mentioned earlier. Additionally the Gadd45 genes regulated by FoxK2 may also form a link to AP1 signaling as GADD45 proteins are upstream activators of the MAPKs JNK and p38 MAPK. Whereas JNK and p38 are direct regulators of various AP1 components required for AP1-dependent transcriptional regulation.

FoxK2 suppresses cell death

Since the absence of FoxK2 leads to the induction of Puma and Noxa (Fig. 5), the cell is shifted towards a pro-apoptotic state. Supporting this is the fact that TGFβ application potently induces caspase 3 cleavage and cell death when FoxK2 is knocked down (Fig. 7). With the identification of specific pro-apoptotic Bcl2 genes, such as Puma and Noxa, we have made a
link between FoxK2 and the apoptotic machinery, providing first steps to uncover the mechanism behind FoxK2 levels and cell death. Possibly mTORC1 activity in the presence of serum is responsible for the attenuation of the effects caused by FoxK2 knockdown, which is also supported by the finding that Noxa is increased after rapamycin treatment (Fig. 5C). Overall, it appears that FoxK2 knockdown has 3 major effects on the cell. Firstly it reduces proliferation, secondly it induces cell death, third a compensatory route involving mTORC1 is activated to dampen the effects on proliferation and cell death. This suggest a central role for FoxK2 in the regulation of cellular proliferation and survival (Fig. 8).

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Literature Cited


