Supplementary materials and methods

Cell culture and transfection

All cells have been described previously[1,2] and have been subcultured for the present study. All cells were maintained in Dulbecco's Modified Eagle's Medium (Cambrex), 10% Fetal Calf Serum, penicillin/streptomycin and 0.05% glutamine. HEK293T, U2OS and A14 cells have been described previously were transfected via the calcium-phosphate method. CHL, Colo829 and WM266.4 cells were transfected with Effectene according to the manufacturer's protocol (Qiagen).

Constructs and RNAi - continued

pMT2-HA-FOXO4-4A was generated by site-directed mutagenesis with the following oligo's: FOXO4-T223A-S226Afwd: 5' ccacccgaaggtgccgctccaacggcccctgtc 3' and FOXO4-T223A-S226Arev: 5' gacaggggccgttggagcggcaccttcgggtgg 3'. As a template we used pMT2-HA-FOXO4-T447/451A[3]. pSuperior-FOXO1/3 and pSuperior-FOXO4 (both against human and mouse) were generated according to the manufacturer's instructions using pSuperior-retro-puro as a template (OligoEngine) and the following oligo's:

FOXO4-1fwd: 5' gatcccggaaatcagtcatatgcagaattcaagagattctgcatatgactgatttccttttta 3', FOXO4-1rev: 5' agcttaaaaaggaaatcagtcatatgcagaatctcttgaattctgcatatgactgatttccgg 3'; FOXO4-2fwd: 5' gatcccgttcatcaaggttcacaacttcaagagagttgtgaaccttgatgaacttttta 3', FOXO4-2rev: 5' agcttaaaaagttcatcaaggttcacaactctcttgaagttgtgaaccttgatgaacgg 3'; FOXO1/3-fwd: 5'

gatcccgtgccctacttcaaggataagttcaagagacttatccttgaagtagggcacttttta 3'; FOXO1/3-rev: 5' agcttaaaaagtgccctacttcaaggataagtctcttgaacttatccttgaagtagggcacgg 3'.

Immunoprecipitation and *in vitro* JNK kinase assays

Immunoprecipitation and GST-pulldown assays were performed as described[1] in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 1% TX-100, 0.5% NaDoC, 5 mM EDTA, 150 mM NaCl, protease and phosphatase inhibitors. For *in vitro* JNK kinase assays HA-FOXO4 or GST-FOXO4ΔDB (which lacks the DNA binding domain, that does not harbor Ser/Thr-Pro sequences and therefore lacks potential JNK phosphorylation sites) were precipitated and processed according to the manufacturer's protocol (Upstate).

Cell cycle distribution by FACS

U2OS cells were transfected with the appropriate plasmids in combination with 250ng GFP-Spectrin. At 36hrs post transfection cells were treated for an additional 24hrs with 250ng/ml nocodazole and processed for FACS analysis as described[4] on a FACScalibur (ABI), using WinMDI v2.9 to analyze data.

Quantitative Real-time PCR

The expression of endogenous $p21^{cip1}$ mRNA in HEK293T cells was examined by reverse transcription of total RNA followed by real-time quantitative PCR (qPCR) on an ABI cycler using Sybr Green (ABI) as described[1], with the following oligonucleotides: $p21^{cip1}$fwd: 5'

ccgaggcactcagaggag 3'; $p21^{cip1}$rev: 5' agctgctcgctgtccact 3' and PBGDfwd: 5'ggcaatgcggctgcaa3' PBGDrev: 5'gggtacccacgcgaatcac3', respectively.

Luciferase assays

Cells were transfected with in triplicate 500ng of the indicated reporter construct and 100ng TK-renilla luciferase. Luciferase activity was analyzed 48 hours post-transfection using a luminometer according to the manufacturer's instructions (Promega).

Mass spectrometry

Input material was digested with trypsin, subtilisin, and/or elastase (Roche). Samples were enriched for phosphorylated peptides using TiO2 microcolumns, as described[1]. Samples were subjected to nanoflow LC (Agilent 1100 series) coupled to a quadrupole time-of-flight tandem mass spectrometer (Micromass Waters). Data were processed and subjected to database searches using MASCOT software (Matrix Science). The identified peptides were confirmed by manual interpretation of the spectra.

Reference List

1. Brenkman, A. B., de Keizer, P. L., van den Broek, N. J., van der, G. P., van Diest, P. J., van der, H. A., Smits, A. M., and Burgering, B. M. The peptidyl-isomerase Pin1 regulates

p27kip1 expression through inhibition of Forkhead box O tumor suppressors. Cancer Res., *68*: 7597-7605, 2008.

2. Gray-Schopfer, V. C., Karasarides, M., Hayward, R., and Marais, R. Tumor necrosis factor-alpha blocks apoptosis in melanoma cells when BRAF signaling is inhibited. Cancer Res., *67*: 122-129, 2007.

3. de Ruiter, N. D., Burgering, B. M., and Bos, J. L. Regulation of the Forkhead transcription factor AFX by Ral-dependent phosphorylation of threonines 447 and 451. Mol.Cell Biol., *21*: 8225-8235, 2001.

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