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FAU expression is essential for proliferation and affects the drug sensitivity of osteosarcoma cells.

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Abstract

The \textit{FAU} gene (Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV)-Associated Ubiquitously expressed gene) was originally identified as a pro-apoptotic gene and its downregulation has been associated with tumourigenicity and drug resistance in tumour cell lines of various origins. The \textit{FAU} mRNA encodes for a fusion product of the ubiquitin-like protein FUBI and the ribosomal protein S30, which is cleaved after a Gly-Gly motif, yielding the mature proteins. It has been demonstrated that the FUBI protein can be conjugated to the pro-apoptotic Bcl-2 family member Bcl-G, which was reported to be essential for the induction of apoptosis by FAU. In contrast, others have reported that FAU and FUBI overexpression has transforming capabilities, whereas FAU and S30 overexpression conferred arsenite resistance. In this study we investigated the function of the \textit{FAU} gene in osteosarcoma and found that \textit{FAU} expression is essential for cell proliferation. Knockdown of \textit{FAU} resulted in a strong decrease of the proliferation rate caused by a G2/M arrest, accompanied by an increase of the checkpoint protein GADD45-alpha. This effect could only be rescued by the ectopic overexpression of the complete \textit{FAU} mRNA, suggesting an essential function for both FUBI and S30. Furthermore, we demonstrated that cells with reduced \textit{FAU} expression showed less sensitivity to treatment with various types of chemotherapeutical drugs, confirming a role of FAU in determining drug sensitivity.
Introduction

Ubiquitination is an important post-translation protein modification involved in the regulation of many cellular processes. Ubiquitin is conjugated to a target protein by formation of an isopeptide bond between the C-terminal glycine of ubiquitin and a lysine in the target protein. A target protein can be (multiple) mono-ubiquitinated or poly-ubiquitinated. Dependent on the ubiquitination status, ubiquitination may lead to altered localization or function of the protein, or can trigger the 26S-proteasome-mediated degradation of the target protein, reviewed recently. A number of ubiquitin-like modifiers have been identified, like SUMO and NEDD8, the functions of which are being gradually unraveled, reviewed in Gareau and Lima (2010) and Watson et al. (2011). One of the least studied ubiquitin-like proteins is FUBI, which is translated as a fusion protein named FAU. The FAU gene encodes a 133 amino acid fusion protein, consisting of the N-terminal ubiquitin-like FUBI protein (74 amino acids) and the C-terminal ribosomal protein S30 (59 amino acids). The FUBI protein contains a C-terminal di-glycine motif, like ubiquitin, enabling it to be conjugated to target proteins. The FUBI protein is ~35% identical to ubiquitin, but, strikingly, lacks almost all internal lysines with the exception of lysine 25, corresponding to lysine 27 in ubiquitin. Not much is known about the function of K27 linkage in ubiquitin, although it has been suggested that all ubiquitin linkages can target proteins for proteasomal degradation. K27 linkage was reported to be assembled by U-box-type 3 E3 ligases under certain stress responses, or by TRAF6 overexpression. Furthermore, the RingB1 E3 ligase promotes self-ubiquitination using mixed a-typical ubiquitin chains, including K27, which are required for its ligase activity towards Histone H2A. Interestingly, the FUBI (Ubi-L) domain of FAU has been reported to bind non-covalently to Histone H2A.

The FAU gene (Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV)-Associated Ubiquitously expressed gene) was identified in two independent studies by functional expression cloning to be a novel apoptosis regulator. Originally, the FAU gene was identified as the cellular homologue of the fox sequence in FBR-MuSV, isolated from a radiation-induced mouse osteosarcoma. The viral fox sequence is expressed as an antisense of the mouse FAU cDNA sequence and was found to posses transforming capabilities, most likely by inactivating endogenous FAU expression. The FAU gene is conserved in higher eukaryotes and expressed in all mammalian tissues.

The FAU antisense sequence inferred resistance to cisplatin and UV-radiation in mouse T-cells. Moreover, overexpression of FAU was shown to induce apoptosis in several cell
lines, revealing a pro-apoptotic function. In these studies the putative distinct functions of FUBI and S30 were not addressed. A possible explanation for the role of FUBI in regulation of apoptosis was initially provided by Nakamura et al. (2003). They showed that the FUBI domain of FAU, also called MNSFbeta, could be conjugated to the pro-apoptotic Bcl-2 family member Bcl-G, thereby inhibiting the ERK cascade. In subsequent studies it was shown that regulation of Bcl-G by FUBI modification is essential for its pro-apoptotic activity, and that FAU expression is frequently downregulated in breast cancer development, which was associated with poor patient survival. Selection for low FAU expression was also observed in ovarian and prostate cancer, correlating with reduced Bcl-G expression. Furthermore, reduction of FAU expression in the A2780 ovarian cancer cell line conferred some carboplatin resistance to these cells. In line with these results, the downregulation of FAU and Bcl-G expression in the prostate cancer cell line 22Rv1 as well as the breast cancer cell line T-47D resulted in decreased sensitivity to UV-C irradiation. These results were recently repeated in a study containing three more human cell lines, and it was shown that FAU overexpression somewhat decreased the number of viable cells. All these studies indicate a tumour suppressor role for FAU in cancer development.

In contrast, in another study, the FAU sequence was shown to protect against arsenic-induced apoptosis. The same authors later showed that overexpression of FAU or its FUBI domain could transform the osteosarcoma cell line HOS to anchorage-independence, whereas the FAU or the S30 subunit alone both induced arsenite resistance. These results would suggest an oncogenic function for FAU.

Furthermore, it was demonstrated that the FAU ubiquitin-like domain is involved in the immune response, although this might be independent from its pro-apoptotic function.

We investigated the role of FAU in the sensitivity of osteosarcoma cell lines to several types of drugs, including arsenic trioxide (ATO). Arsenic trioxide is used to induce apoptosis in acute promyelocytic leukemia (APL) and was also reported to induce apoptosis in solid cancer cell lines. Furthermore, it was shown that arsenic trioxide treatment induced apoptosis via p73, which could be enhanced with MEK1 inhibitors. Recently, it has been reported that ATO treatment reduced motility, migration and invasiveness in the osteosarcoma cell lines HOS and MNNG, probably by the inhibition of phosphorylation of MAPK family members ERK and MEK. Several groups report that in other osteosarcoma cell lines, such as MG-63 or SaOS-2, ATO treatment successfully can induce apoptosis and act synergistically with other drugs.
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On the other hand, exposure to low concentrations of ATO has been suggested to act carcinogenic \(^{34-36}\), possibly by the observed activation of the p38-MAPK, ERK1/2 and Akt pathways upon low dose treatment with ATO \(^{37}\).

To investigate FAU functions on drug sensitivity, we performed both knockdown of FAU and overexpression of FAU, FUBI or S30 and assessed the effects of drug treatments. Surprisingly, we found a significant growth inhibiting effect of FAU knockdown, which could only be rescued by concomitant overexpression of the whole FAU sequence. Furthermore, we observed decreased sensitivity for arsenic trioxide, cisplatin and doxorubicin in cells with decreased FAU levels. On the other hand, overexpression of FAU and FUBI increased the sensitivity for these drugs.

Material & Methods

Cell Culture and Reagents
U2OS, OST and OSA cells were maintained in RPMI supplemented with 10% FBS and antibiotics. Cells were treated with arsenic trioxide, doxorubicin, cisplatin, Actinomycin D (all from Sigma-Aldrich, St Louis, MO, USA), MEK1/2 inhibitor U0126 (Calbiochem), MEK1 inhibitor PD98059 (Calbiochem), RITA (Gift from Galina Selivanova) and Nutlin-3 (Cayman Chemical, Ann Arbor, MI, USA) for the indicated times and concentrations.

Plasmids, Lentiviral Transduction
A FAU cDNA clone was obtained from Origene Technologies Inc. (Rockville, MD, USA). The coding sequences of FAU, FUBI or S30 were amplified, with the use of 5' primers containing a BamHI and 3' primers containing an XbaI restriction site. The resulting fragment was cloned into a pcDNA3.1 expression vector containing an HA-tag such that the HA-tag is N-terminal of the FAU, FUBI or S30 coding region. Lentiviral expression vectors for HA-FAU, HA-FUBI and HA-S30 were made by cloning the corresponding cDNA sequences into pLV-CMV-bc-puro using NdeI and Nhel restriction sites.

Other lentiviral shRNA expression vectors were the non-targeting shRNA expression lentivirus shc002 (Sigma MISSION Library; Sigma-Aldrich), shRNA targeting human p53 \(^{38}\) or mouse mdmx \(^{39}\) as a control. The construction of lentiviral vectors expressing specific shRNAs and the production of lentivirus particles have been described recently \(^{39}\).

Proliferation assay, Colony Forming assay, FACS analysis
For growth assays, 2000 cells were seeded in triplicate in a 96-wells plate; 24 hours after seeding, cells were treated with the indicated drugs for the indicated times. To measure cell survival, cells were incubated with WST-1 reagent (Roche Biochemicals, Indianapolis, IN) for 1-2 hours followed by quantification with a plate reader.
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(Victor3 Multilabel Counter 1420-042, Perkin-Elmer) measuring absorbance at 450 nm. The growth inhibitory effect of the drug was analyzed with the use of CompuSyn software.

For colony forming assays 3000-4000 cells were seeded in duplicate per condition in 6-wells plates; cells were treated for three days with 3 µM ATO or mock treated and fixated and stained with Giemsa reagens eight days after seeding. Colonies were quantified with the Odyssey Infrared Imager and Odyssey 2.1 analysis software (LI-COR Biosciences, Lincoln, Nebraska USA).

For FACS analysis 100,000 cells were seeded in 6-cm dishes 24 hours before the indicated treatment. Cells and medium were harvested after 48 hours of treatment, fixated and analyzed on the Flow cytometer (Beckton Dickinson, Franklin Lakes, NJ USA) using FACS DIVA software after incubation in PBS with 50 µg/ml propidium-iodide (PI) and 50 µg/ml RNase.

RNA isolation, Reverse Transcription, Q-RT-PCR

RNA was isolated from cells using the SV Total RNA Isolation System (Promega) according to the manufacturers’ protocol. cDNA was made from 1 µg of total RNA in an Reverse Transcriptase reaction containing ImPromII Reverse Transcriptase, RNasin RNase inhibitor, MgCl2 and 5x RT-Buffer (Promega). Quantification of mRNA expression was performed by Quantitative-real-time PCR, in an ABI 7900HT thermocycler, using the FastStart Universal SYBR Green Master (ROX) mix (Roche). Relative mRNA quantities were calculated using an internal standard curve, made from a dilution series of the pooled samples, using the SDS2.3 analysis program (Applied Biosystems). Expression levels of the mRNAs were normalized to the geometric mean of the control genes CAPNS1, TBP, SRPR. Primer sequences are available in Supplemental Information.

Protein extraction, Western Blotting and Antibodies

Cells were lysed in Giordano 250 buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA), supplemented with a mixture of protease- and phosphatase inhibitors. Protein content was determined using Bradford Reagent (Biorad) and equal amounts of protein were separated by SDS-PAGE. Proteins were transferred onto polyvinyldene difluoride membranes (Immobilon-P, Millipore). Membranes were blocked in Tris-buffered saline, 0.2% Tween-20 (TBST) containing 10% non-fat dry milk, and subsequently incubated with the appropriate primary antibody diluted in TBST containing 10% milk. Membranes were washed three times with TBST and incubated with HRP-conjugated secondary antibody (Jackson Laboratories). Bands were visualized by enhanced chemiluminescence (Super Signal; Pierce) followed by detection with the ChemiGenius XE3 system (Syngene, Cambridge, UK) or X-ray film (Fujifilm).

Antibodies used in this study were anti-HA.11 monoclonal mouse antibody (Covance), rabbit polyclonal anti-USP7 (A300-033A, Bethyl Laboratories), anti-S30 rabbit polyclonal antibody (405, gift of Dr. J.C. Marine), anti-B23 mouse monoclonal antibody NA24 (Santa Cruz), anti-phospho-ERK1/2 monoclonal mouse antibody (Sigma), anti-ERK1/2 rabbit polyclonal antibody (Sigma), anti-phospho-Akt-S473 rabbit polyclonal antibody (Cell Signaling), anti-Akt1/2 rabbit polyclonal antibody (Upstate), anti-phospho-T246 PRAS40 rabbit polyclonal antibody (Cell Signaling), anti-cyclin D1 rabbit polyclonal antibody (Santa Cruz) and anti-p27 rabbit monoclonal antibody (Epitomics). Secondary antibodies goat-anti-mouse-HRP and goat-anti-rabbit-HRP were obtained from Jackson Laboratories.

Immunofluorescence:

Cells were seeded onto coverslips 24 hours pre-fixation. Cells were washed with PBS before fixation with 4% paraformaldehyde for 10 min at room temperature (RT). Cells were subsequently washed with PBS, permeabilized in PBS with 0.2 % Triton X-100 solution for 10 min at RT, washed with PBS and washed with PBS containing 0.05% Tween-20. Cells were blocked with PBS containing 0.05% Tween-20 and 5% Normal Goat Serum (NGS) for 1 hour at RT, incubated with the indicated primary antibodies, diluted in PBS/Tween-20/NGS, for 1.5 hours. After washing, cells were incubated for 30 min with goat anti-mouse-Rhodamine secondary antibody (Jackson) diluted 1:100 in PBS/Tween20/NGS. After washing, coverslips were mounted onto microscope slides using DAPI-DABCO mounting solution, containing 90% glycerol, 10% 0.2M Tris-HCl pH8.0, 2.3% DABCO (1,4 Diazabicyclo [2,2,2] octane) and 0.002% DAPI.
Results

FAU expression is essential for cell proliferation

To assess a putative role of FAU expression in drug sensitivity of osteosarcoma cell lines, we performed both FAU knockdown- and overexpression experiments. Initially, we performed a knockdown of FAU, with 2 different lentiviral knockdown constructs, in 3 osteosarcoma cell lines, U2OS, OST and OSA, which have slightly varying basal FAU mRNA levels (Fig. 1A). Knockdown resulted in at least a 50% decrease in FAU mRNA levels (Fig. 1A). Strikingly, in all three cell lines, knockdown of FAU resulted in a marked growth inhibition with both knockdown vectors (Fig. 1B and C).

To investigate whether the observed inhibition of growth is indeed caused by the reduced FAU expression, we tried to rescue the knockdown effect by overexpression of FAU, FUBI or S30. To that end, U2OS cells were transduced with lentiviral vectors expressing HA-FAU, HA-FUBI or HA-S30 or a control vector, and stably transduced cells were obtained by puromycin selection. Subcellular localization of the exogenously expressed proteins was investigated with by immunofluorescence. HA-FAU was found to be present in the nucleus, including the nucleoli (Suppl. Fig. 1A). HA-S30 was predominantly localized in the nucleoli, as demonstrated by co-localization with the nucleolus-specific protein B23 (Suppl. Fig.1B). HA-FUBI was localized throughout the cell, both nuclear and cytoplasmic; however, patterns were strongly variable and might be determined by e.g. cell cycle phase. (Suppl. Fig. 1A). Not all cells transduced with HA-FUBI or HA-S30 lentiviruses detectably expressed the corresponding protein as determined by immunofluorescence, whereas virtually all HA-FAU transduced cells do express HA-FAU. Expression of endogenous FAU, FUBI or S30 was not detectable with the available antibodies; neither by immunofluorescence nor by western blotting.

Subsequently, FAU was knocked down with the same lentiviral knockdown constructs mentioned above, which target endogenous FAU mRNA in its coding region, in the S30 part of the sequence. Consequently, exogenous FAU and S30 will also be targeted for shRNA-mediated degradation upon transduction with these knockdown constructs, whereas exogenous FUBI should not be affected.

The FAU knockdown efficiency in all 4 stable U2OS cell lines is shown in Supplemental Figure 1C. Depicted are the relative mRNA quantities determined with the use of primer sets for respectively endogenous FAU, total FUBI and total S30. At the mRNA level, both FAU and FUBI are highly expressed, whereas S30 is somewhat lower. Both FAU and S30 are decreased upon FAU knockdown, whereas FUBI mRNA levels remain constant. Nevertheless, compared to control cells, the FAU overexpressing cells still have much
higher FAU mRNA levels after FAU knockdown, and even S30 overexpressing cells still retain about 5-10x higher S30 mRNA levels compared to control after knockdown of FAU.

At the protein level (Suppl. Fig. 1D) both HA-FAU and HA-S30 expression are decreased upon FAU knockdown, although the HA-FAU expression is still well detectable. HA-FUBI expression is not significantly affected, as expected. In the HA-FAU cells we also detect a band of the same size as HA-FUBI. This most likely indicates that FAU is cleaved at the GG-motif, which essentially yields an HA-FUBI protein (detected with anti-HA antibody) and the untagged S30 protein, which is not detected. Interestingly, a smear of high molecular species is detected in both HA-FUBI and HA-FAU expressing cells, which most likely consists of FUBI-conjugates. Since S30 is lower expressed, a longer exposure of the blot is shown. Probably therefore some aspecific bands were detected in both Ctrl and S30 cells, but the intensity is much lower than the FUBI-conjugates detected in the FUBI and FAU cells.

Immunofluorescence on cells that had FAU knocked down, confirmed the results found on western and RNA; HA-FAU overexpressing cells showed a slightly decreased signal for FAU and nucleolar staining was more obvious. HA-FUBI expression and localization seemed to be unaffected, whereas the specific S30 signal in HA-S30 cells was significantly reduced (Suppl. Fig. 1A).

Although FAU has been reported to be a pro-apoptotic gene and, therefore, a growth reduction might be expected, overexpression of both FAU and FUBI did not significantly change the growth rate of the cells, whereas cells with S30 overexpression showed a somewhat reduced proliferation rate (Fig. 1D).

In contrast, knockdown of FAU resulted in growth inhibition in control-, HA-FUBI- and HA-S30 overexpressing cells, but was almost completely rescued by HA-FAU overexpression (Fig. 1E). Furthermore, we also observed partial rescue of the growth inhibitory effect upon FAU knockdown by HA-FAU overexpression in a long term growth assay, a colony forming assay (Fig. 1F).

FACS analysis was performed to find an explanation for the growth-inhibition seen upon knockdown of FAU (Suppl. Fig. 2A). The most striking change observed is a strong increase of the number of cells in G2/M phase upon FAU knockdown, with concomitant reduced G1- and S-phase cells. Furthermore, a slight increase of cells in SubG1 is observed, suggesting some induction of apoptosis. Overexpression of FAU, FUBI or S30 did not significantly change the cell cycle profile (Suppl. Fig. 2A). Importantly, HA-FAU overexpression largely rescued the cell cycle effects seen upon FAU knockdown. The constitutive expression of FUBI or S30 could not or only slightly rescue the cell cycle effects. However, since particularly the levels of HA-S30 are lower than the HA-FAU
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Figure 1. FAU expression is essential for cell proliferation

U2OS, OST and OSA cells were lentivirally transduced with control-, or two different FAU knockdown constructs. Cells were seeded for proliferation assays and RNA isolation 24 hours after infection. RNA extracts were isolated at day 4 post-infection. Expression levels of FAU mRNA were quantified using q-RT-PCR and are
shown as normalized relative mRNA levels (A). Metabolic activity as a measure of proliferation was measured at the indicated days and displayed as relative growth to day 0 (B) or as relative survival at day 2 compared to control cells (C). U2OS cells were lentivirally transduced with either control vector or expression vectors for HA-FUBI, HA-S30 or HA-FAU. Stable cell lines were established by puromycin selection and survival relative to control cells was measured 3 days after seeding using WST-1 proliferation assays (D). Stable cell lines were lentivirally infected with either control or two different FAU knockdown constructs. Cells were seeded for WST-1 proliferation assays and colony formation assays 48 hours after infection. Relative survival of transduced cells was measured 3 days after seeding (E). Colony formation of stable control or HA-FAU expressing cells transduced with either control or FAU knockdown was quantified 10 days after seeding and displayed as relative colony formation compared to the corresponding control cells (F).

expression after FAU knockdown, the rescue effect might be attenuated compared to the effect of FAU overexpression. However, based on the observations in short and long term proliferation assays and the results of flow-cytometry, it seems that the expression of the complete FAU sequence is needed to rescue the growth inhibiting effect of FAU knockdown.

**FAU expression enhances sensitivity for ATO treatment in U2OS cells.**
To assess a putative effect of FAU levels on the sensitivity to arsenic trioxide (ATO) in osteosarcoma cells, we treated the above described stable cell lines for 24 hours with 3 µM ATO and cell survival was determined. Although the control cells show a relative survival of only 25% after 72 hrs, cells that overexpress FAU show an even lower relative survival (Fig. 2A). Moreover, as illustrated in the growth curves (Fig. 2B), ATO-treated control cells are just growth arrested, whereas the number of ATO-treated FAU overexpressing cells actually decreases, suggesting cell loss through apoptosis. Furthermore, cells with FAU knockdown, which already have a somewhat reduced growth rate, show almost no further reduction upon ATO treatment (Fig. 2C). Cells that have FAU overexpression and FAU knockdown combined, show an intermediate survival (Fig. 2B). FACS analysis showed an increased number of control cells in G2/M phase after 48 hours of ATO treatment, which was not significantly influenced by FAU overexpression. In FAU knockdown cells, ATO treatment did not further increase the amount of cells in G2/M arrest (Supp. Fig. 2B). Even more, in a colony formation assay, arsenic trioxide treatment could not further reduce colony formation after FAU knockdown, whereas cells with FAU overexpression showed a higher sensitivity for this treatment (Fig. 2D). FAU overexpression also sensitizes p53-knockdown cells for ATO treatment (Fig. 2E), suggesting that the increased ATO sensitivity induced by FAU overexpression is not dependent on p53. However, when cells were treated with higher concentrations of this drug, to induce an apoptotic response, no differences were observed between control-, FAU- or FUBI-overexpressing cells, whereas S30 overexpressing cells seemed to be slightly more resistant (Fig. 2F). Interestingly, cells with reduced FAU levels were also more resistant to the high ATO treatment (Fig. 2G).
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Figure 2. FAU expression enhances sensitivity for ATO treatment, independent of p53.
Stable U2OS cells expressing a control vector or HA-FAU were lentivirally infected with either a control- or two different FAU knockdown vectors. Cells were seeded for WST-1 proliferation assays and colony formation assays 2 days post-infection and treated with 3 µM ATO for 24 hours or mock treated one day after seeding. Proliferation was measured using WST-1 and displayed as relative survival at day 3 compared to the corresponding mock-treated cells (A). Growth curves of mock- or ATO (3 µM 24 hours) treated control and HA-FAU cells are depicted in (B), growth curves of mock- or ATO (3 µM 24 hours) treated control cells or cells that have decreased FAU levels due to expression of one of two different FAU knockdown constructs are depicted in (C). Colony formation of ATO (3 µM 24 hours) treated control- or FAU-knockdown cells is displayed as relative colony formation.
compared to the corresponding mock treated cells (D). Stable U2OS cell lines expressing either a control vector or an expression vector encoding HA-FAU or short-hairpin RNA’s against p53, or both, were seeded for WST-1 proliferation assays. One day after seeding cells were treated with 3 µM ATO for 24 hours or mock treated. Relative survival of ATO treated cells at day 3 after treatment is depicted in (E). U2OS cells were lentivirally infected with a control vector or an expression vector encoding HA-FUBI, HA-S30 or HA-FAU or either a control-knockdown vector or two different FAU knockdown vectors, and seeded for WST-1 proliferation assays four days post-infection. One day after seeding the cells were treated with the indicated concentrations of ATO for 24 hours or mock-treated. Relative survival of control-, HA-FUBI-, HA-S30 and HA-FAU cells at day 2 after treatment is depicted in (F). Relative survival of control- and FAU-knockdown at day 2 after treatment is depicted in (G).

FAU increases ATO sensitivity of U2OS cells, independently from the ERK1/2 pathway.

Since it has been reported that inhibition of the MEK/ERK1/2 pathway increases ATO sensitivity 27-29, we treated control- and FAU-overexpressing cells with arsenic trioxide in the presence or absence of a MEK inhibitor, U0126. Both control and FAU overexpressing cells showed increased ATO sensitivity when co-treated with the MEK inhibitor (Fig. 3A). Indeed, we find that ATO treatment induces phosphorylation of ERK1/2 in both control and HA-FAU overexpressing cells (Fig. 3B). ERK1/2 activation actually functions as a survival signal, thereby attenuating the effect of ATO. Addition of MEK inhibitors U0126 and PD98059 reduced both basal (Fig. 3C, lower panel) and ATO-induced ERK1/2 phosphorylation (Fig. 3C, upper panel). Interestingly, we observed an increase of ERK1/2 phosphorylation upon FAU knockdown, which could still slightly be enhanced by ATO treatment and which was found sensitive to co-treatment with MEK inhibitors (Fig. 3C). A combination treatment with ATO and MEK inhibitors slightly reduced the survival of the FAU knockdown cells, whereas single ATO treatment had no effect (Fig. 3D). Interestingly, treatment with each MEK inhibitor alone seemed to enhance the growth of the FAU knockdown cells somewhat, whereas no effect was observed in the control cells, suggesting that the enhanced ERK1/2 activation in these knockdown cells might have an anti-proliferative function, as has been reported before 41,42. Altogether, these results suggest that FAU expression modulates the sensitivity of cells for ATO, independent of ERK1/2 activation. However, the activation of ERK1/2 seems to attenuate the growth inhibiting effect of ATO treatment, independent of the FAU levels. Furthermore, the FAU knockdown experiments suggest that endogenous levels of FAU inhibit basal phosphorylation of ERK1/2, as was published before 16, although we did not observe decreased phospho-ERK levels in FAU overexpressing cells, suggesting that endogenous levels are sufficient for this function of FAU (Fig. 3B).
Knockdown of FAU and ATO treatment induces gadd45-alpha mRNA expression.

Upon FAU knockdown and also upon ATO treatment we observed an increase of p-ERK1/2, as well as a marked G2/M arrest (Fig. 3C and Supp. Fig. 2B). A candidate protein involved in the biological response to ATO treatment and FAU manipulation is GADD45-alpha. GADD45-alpha is a checkpoint protein, arresting cells at G2/M phase by inhibiting the activity of cyclinB/p34cdc2 complex in response to stress \(^{43}\) and it has been reported that the transcription of the gadd45-alpha gene can be regulated independently from p53 by the MAPK pathways \(^{44}\). Furthermore, it was shown that both JNK1 and ERK1/2 activation could enhance the DNA damage induced GADD45-alpha expression, by interacting with transcription factors such as Oct-1 and NF-YA that bind to OCT-1 and CAAT motifs in the gadd45-alpha promoter \(^{44,45}\).

Indeed we found increased gadd45-alpha mRNA expression in cells with reduced FAU levels (Fig. 4A), which might, at least partly, explain the G2 arrest seen upon FAU knockdown. This increase could partially be rescued by FAU overexpression, although it did not affect basal gadd45-alpha expression. ATO treatment also induced gadd45-alpha levels, p53-independently. ATO did not further increase gadd45-alpha in FAU knockdown cells, which already have increased levels (Fig. 4B), which might suggest that ATO and FAU regulate gadd45-alpha via the same pathway. FAU overexpression did not affect the ATO-induced expression of gadd45-alpha (Fig. 4C), indicating that the increased sensitivity of FAU overexpressing cells is not caused by reduced gadd45-alpha induction. Even though these data correlate well with the regulation of phospho-ERK shown in Figure 3, it seems unlikely that the phosphorylation of ERK1/2 is the primary trigger for the induction of gadd45-alpha. On the contrary, the use of MEK inhibitors together with ATO even further enhanced the expression of gadd45-alpha and slightly stimulated the growth inhibitory effect of ATO treatment (Fig. 4C and Fig. 3A). Together, these results suggest that both ATO treatment and reduced FAU expression lead to p53-independent induction of gadd45-alpha, resulting in G2/M arrest, possibly via activation of MAPK pathways, although the observed ERK1/2 activation appears not to be required.

It has also been reported that levels of active, phosphorylated-Akt are transiently upregulated upon ATO treatment but decrease at later time-points \(^{46}\). Prevention of the transient activation of Akt strongly increased the apoptotic response. Importantly, Akt is inactivated upon ATO treatment at later time points through induction of c-Cbl proteins. This inactivation of Akt was found to be important for the apoptotic response upon ATO treatment \(^{46,47}\). Moreover, in another study it was found that the inhibition of Akt activation enhanced the ERK1/2 phosphorylation after certain types of stress \(^{48}\). Therefore, we analyzed whether FAU overexpression would affect the Akt activation by ATO treatment,
by investigation of phospho-Akt levels and the expression of genes whose expression can be regulated via Akt activation. In both control and FAU cell lines we find Akt inactivated at later time points upon ATO treatment (Fig. 4D).

Figure 3. FAU increases ATO sensitivity independent from the ERK1/2 pathway.
Stable U2OS cells expressing either a control vector or a HA-FAU expression vector were seeded for a WST-1 proliferation assay, 24 hours after seeding cells were treated with 3 µM ATO or 3µM ATO and 10 µM U0126 for 8 hours or mock treated. Relative survival compared to mock-treated corresponding cells 48 hours after start of treatment is displayed in (A). Stable control-vector or HA-FAU expressing U2OS cells were treated with 3 or 5 µM ATO for 24 hours or mock treated, after which protein extracts were isolated. Protein extracts were analyzed with immunoblotting using the indicated antibodies. USP7 expression was used as a loading control (B). U2OS cells were lentivirally infected with either a control- or two different FAU knockdown vectors. At day 2 post-infection cells were treated with 3 µM ATO, 10 µM U0126, 15 µM PD98059 or a combination of ATO and one of both inhibitors for 24 hours, or mock treated. Protein extracts were isolated and analyzed with immunoblotting using anti-phospho-ERK1/2 and anti-ERK1/2 antibodies (C). Relative survival compared to mock-treated corresponding cells 96 hours after start of treatment is displayed in (D).
Figure 4. Knockdown of FAU and ATO treatment induces gadd45-alpha mRNA expression.

Stable U2OS cells expressing a control vector or HA-FAU were lentivirally infected with either a control- or two different FAU knockdown vectors. RNA extracts were isolated at day 6 post-infection and GADD45-alpha mRNA levels were analyzed using q-RT-PCR and are shown as normalized relative mRNA levels (A). Stable U2OS cells expressing a control- or p53-knockdown vector were lentivirally infected with either a control- or two different FAU knockdown vectors. At day 4 post-infection cells were treated with 3 µM ATO for 48 hours or mock treated and RNA extracts were isolated. Expression levels of GADD45-alpha mRNA were analyzed using q-RT-PCR and are shown as normalized relative mRNA levels (B). Stable U2OS cells expressing a control vector or HA-FAU were treated with 3 µM ATO, 3 µM ATO together with 10 µM U0126 or mock-treated for 24 hours. Total RNA was isolated and mRNA levels of GADD45-alpha were analyzed using q-RT-PCR and are shown as normalized relative mRNA levels (C). Stable U2OS cells expressing a control vector or HA-FAU were treated with 3 µM ATO for the indicated times, after which protein extracts were isolated and analyzed with immunoblotting, using the indicated antibodies. USP7 expression was used as a loading control (D).
FAU overexpression does sensitize U2OS cells for cisplatin and doxorubicin treatment.

To study the effect of FAU expression on the response to other drugs, we treated control or FAU overexpressing U2OS cells with various concentrations of cisplatin and doxorubicin, enabling us to calculate the effective dose for a certain affected fraction (Fig. 5A and Table 1). FAU overexpressing cells were slightly more sensitive for both cisplatin and doxorubicin; differences were the clearest when concentrations were used that affected more than 90% of the cells. Results were confirmed in colony forming assays, when the cells were treated with low concentrations of either cisplatin or doxorubicin for 24 hours. Again, FAU overexpressing cells were relatively more sensitive (Fig. 5B). However, using these concentrations, it seemed that the cells just arrest their growth but did not show an apoptotic reaction, as no floating cells were observed. Indeed, FACS analysis showed that 24 and 48 hours treatment with 1 µM cisplatin resulted in a large increase in number of G2/M cells of both control- and FAU-overexpressing cells (Fig. 5C). Importantly, hardly any increase in sub-G1 fraction was observed, indicating that this low dose of cisplatin does not notably induce apoptosis at this time-point. Some increase of the sub-G1 fraction was observed when the cells were treated with 0.5 µM doxorubicin (Fig. 5C), although this did not correlate with the increased sensitivity of the FAU overexpressing cells (Supp. Fig. 3A).

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<tr>
<th>Effective dose [nM]</th>
<th>CisPlatin</th>
<th>Doxorubicin</th>
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<tbody>
<tr>
<td>Fa</td>
<td>Ctrl</td>
<td>FAU</td>
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U2OS cells stably transduced with either a control vector or HA-FAU expression vector were seeded for WST-1 proliferation assays and colony formation assays. Next day the cells were treated with the indicated concentrations of cisplatin or doxorubicin for 24 hours or mock-treated. Proliferation was measured at day 3 after treatment and effective doses were calculated using the CompuSyn programme. Fa = affected fraction. For cisplatin, \( r = 0.99422 \), and for doxorubicin treatment \( r = 0.99337 \).

Reduction of FAU increases the resistance of U2OS cells to various drugs

Confirming the effects that were observed in cells that overexpressed FAU, cells with reduced \( FAU \) levels were more resistant to cisplatin and doxorubicin. U2OS cells with reduced \( FAU \) levels were in a short-term growth assay slightly more resistant to cisplatin (1
Effects of FAU expression in osteosarcoma cells

µM) and significantly more resistant to doxorubicin (0.5 µM) (Fig. 5D). Similar effects were observed in long-term colony assays, in which the growth inhibition by the drugs was more severe. Even so, cells with reduced FAU levels the inhibitory effect of the drug treatment on colony formation was less (Fig. 5E). When cells were treated with higher concentrations cisplatin and doxorubicin, the differences between FAU overexpressing cells and control cells were diminished, but FAU knockdown cells remained more resistant (Supp. Fig. 3B and C). Similarly, when the cells were treated with other drugs, such as Actinomycine D, RITA and Nutlin-3, we observed a reduced sensitivity for the FAU knockdown cells (Supp. Fig. 3D).

Discussion

The FAU gene was reported to exhibit tumor suppressing and pro-apoptotic activities, although also oncogenic functions have been designated to this gene.

It has been shown that the regulation of Bcl-G by FAU is required for its apoptotic functions. In several tumor types the expression of both genes was found to be reduced, correlating with tumor progression, worse patient survival and increased drugs resistance. On the other hand, FAU overexpression has been associated with increased arsenic trioxide resistance and transformation in HOS osteosacoma cells. In that study, the S30 domain was responsible for the increase ATO resistance and the FUBI domain for the transforming ability. Furthermore, FUBI-conjugated Bcl-G was shown to inhibit the MAPK pathway, by inhibiting the phosphorylation of ERK1/2.

In this study, we investigated the role of FAU on the proliferation and drugs resistance in osteosarcoma cell lines. We found a clear growth inhibition, marked by a strong G2/M arrest, as a result of FAU knockdown, which could only be fully rescued by the expression of a complete FAU RNA. In a recently published study, FAU knockdown did not lead to reduced cell viability or apoptosis, however, analyses of cell-cycle progression and growth rate were not performed.

An increase in ERK1/2 phosphorylation was observed upon FAU knockdown, which is consistent with findings that FAU is involved in the inhibition of the MAPK pathway. The growth inhibition caused by FAU knockdown might be the result of GADD45-alpha induction, since we observed increased gadd45-alpha mRNA levels upon FAU knockdown, which could partly be rescued by FAU overexpression. This checkpoint protein can be induced by MAPK activation and induces G2/M arrest. Given the observed enhanced ERK1/2 activity in these knockdown cells and some rescue of the growth inhibition by
Figure 5. FAU overexpression sensitizes cells for cisplatin and doxorubicin treatment.
Stable U2OS cells expressing a control vector or HA-FAU were seeded for WST-1 proliferation assays and colony formation assays. At day 1 after seeding, cells were treated with the indicated concentrations cisplatin or doxorubicin for 24 hours or mock-treated. Proliferation was measured at day 3 after treatment and displayed as relative survival compared to mock-treated corresponding cells (A). Colony formation of control- and HA-FAU cells is displayed as relative colony formation compared to the corresponding mock-treated cells (B). FACS analysis was performed with cells that were treated with cisplatin (1 µM), doxorubicin (0.5 µM) or mock-treated for 48 hours. Indicated are percentages of cycling cells in G1, S or G2/M phase and percentages of single cells in SubG1 (C). U2OS cells were lentivirally infected with either a control- or two different FAU knockdown vectors. At day four post-infection cells were seeded for WST-1 proliferation assays and colony formation assays. One day after seeding, cells were treated with the indicated concentrations cisplatin or doxorubicin for 48 hours or mock treated. Proliferation was measured at day 2 after treatment and displayed as relative survival compared to mock-treated corresponding cells (D). Colony formation of control- and HA-FAU cells, treated for 48 hours with the indicated concentrations and drugs, is displayed as relative colony formation compared to the corresponding mock-treated cells (E).
MEK inhibitors, the growth inhibition might be a direct consequence of an activated MEK/ERK cascade. Prolonged ERK1/2 activation has been reported to cause cell cycle arrest and even apoptosis in some circumstances. In contrast to the reported pro-apoptotic function of FAU in other cells, overexpression of FAU did not influence growth of U2OS cells, nor did it inhibit the activation of ERK1/2. Probably an increase in FAU levels only is not sufficient to induce apoptosis. To exert a pro-apoptotic effect, FUBI needs to be conjugated to Bcl-G, and Bcl-G levels might be limiting. It is more likely that simultaneous overexpression of both Bcl-G with FAU or FUBI is needed to induce apoptosis. The pro-apoptotic function of FAU is more pronounced when cells are treated with drugs that induce an apoptotic response. Upon various drug treatments, Bcl-G levels will also increase, for instance due to p53 activation. Under these conditions, overexpression of FAU will co-operate with the induced Bcl-G levels, which might explain the observed increased drug sensitivity.

Indeed, we find that cells with enhanced FAU levels show increased sensitivity to p53-inducing treatments with ATO, cisplatin, or doxorubicin. Importantly, cells with decreased FAU levels were found to be more resistant to these drugs, again indicating that FAU levels are affecting the response to various drugs.

Interestingly, in p53 knockdown cells FAU overexpression did have a sensitizing effect comparable to control cells, indicating a p53-independent mechanism. Since Bcl-G is a p53 target gene, it suggests that more mechanisms are involved in the FAU-mediated drug sensitivity than only the p53-dependent induction of Bcl-G alone. It would, therefore, be interesting if FAU overexpression still sensitizes these p53-negative cells for drug treatments when Bcl-G levels are reduced. It has been reported that Bcl-G knockdown decreases FAU- and UV-induced apoptosis. However, in these experiments just knockdown of Bcl-G resulted in increased basal apoptosis, whereas the total amount of apoptotic cells after FAU transfection or UV treatment was not significantly decreased. So, it is very difficult to interpret these results.

Strikingly, we found some similarities between the effects observed upon FAU knockdown and ATO treatment. ATO treatment induced ERK1/2 phosphorylation, which could be prevented by MEK inhibitors, thereby enhancing the growth inhibiting effect of ATO. These results suggest that ERK activation is pro-survival in this cell system. Depending on the cells used, activation of ERK by ATO has been reported to inhibit or to enhance ATO-induced apoptosis. Arsenic trioxide induced gadd45-alpha expression, which has also been reported to be regulated by JNK and negatively, by NF-κB. FAU knockdown cells also showed induced p-ERK1/2 levels and increased gadd45-alpha mRNA expression,
suggesting again that FAU regulates the activation of some players in the MAPK pathways and might play a role in the equilibrium between proliferative and growth inhibiting factors. FAU expression might be required for optimal proliferation under normal conditions, whereas upon certain types of cellular stress FAU expression might stimulate the apoptotic response. In this model, apparently contrasting results reported previously might be explained. The transforming activity of FAU and FUBI in HOS cells\textsuperscript{23}, can be explained by the proliferative effects of FAU under normal conditions, whereas its tumor suppressing role is activated under stress conditions.

In conclusion, in this study we show that FAU expression is needed for the normal proliferation of osteosarcoma cells, and that FAU levels influence the sensitivity to several types of drugs, including some that are frequently used in the clinic for osteosarcoma treatment.
Effects of FAU expression in osteosarcoma cells

References


Supplemental Figure 1. Overexpression of HA-tagged FAU, FUBI and S30 in U2OS cells.
U2OS cells were lentivirally transduced with either control vector or expression vectors for HA-FUBI, HA-S30 or HA-FAU. Stable cell lines were established by puromycin selection. Stable cell lines were lentivirally infected with a control- or a FAU knockdown vector. Localization and expression of the exogenous HA-tagged proteins was determined by immunofluorescence with anti-HA antibodies. DAPI staining was used to visualise nuclei (A). Co-localisation of exogenous HA-S30 and the nucleolar protein B23 was visualised in stable control or HA-S30 expressing U2OS cells using immunofluorescence with anti-B23 and anti-S30 antibodies. DAPI staining was used to visualise nuclei and merged images are shown in the most right panel (B). Protein and mRNA extracts of stable control-, HA-FAU, HA-FUBI and HA-S30 expressing U2OS cells were isolated 6 days after lentiviral infection with control- or FAU knockdown vectors. Expression levels of endogenous FAU, total FUBI and total S30 mRNA were analyzed using q-RT-PCR and are shown as normalized relative mRNA levels (C). Protein extracts were analyzed with immunoblotting using an anti-HA antibody, USP7 expression was used as loading control (D).
Supplemental Figure 2. FAU overexpression rescues FAU-knockdown induced G2/M arrest; FAU knockdown cells are resistant to ATO treatment.

Stable U2OS cells expressing a control vector or expression vectors for HA-FAU, HA-FUBI or HA-S30 were lentivirally infected with either a control- or two different FAU knockdown vectors. At day 6 post-infection cells were harvested for FACS analysis (A). U2OS cells were lentivirally infected with a control vector, an HA-FAU expression vector or a vector expressing a short hairpin RNA against FAU. At day 4 post-infection, cells were treated with 3 µM ATO for 48 hours or mock treated and harvested for FACS analysis (B). Indicated are percentages of cycling cells in G1, S or G2/M phase and percentages of single cells in SubG1.
Supplemental Figure 3. Reduction of FAU levels decreases sensitivity of U2OS cells for various drugs at high concentrations.

U2OS cells stably expressing a control vector or HA-FAU were seeded for WST-1 proliferation assays. Next day, cells were treated with the indicated concentrations cisplatin or doxorubicin for 24 hours or mock treated. Proliferation was measured 48 hrs later and displayed as relative survival compared to mock-treated corresponding cells (A). U2OS cells were lentivirally infected with either a control vector or an expression vector encoding HA-FAU and seeded for WST-1 proliferation assays four days post-infection. One day after seeding, cells were treated with the indicated concentrations of cisplatin or doxorubicin and cell viability was determined 48 hrs later and displayed as relative survival compared to mock-treated corresponding cells (B). U2OS cells were lentivirally infected with either a control-knockdown vector or two different FAU knockdown vectors, and seeded for WST-1 proliferation assays four days post-infection. One day after seeding, cells were treated with the indicated concentrations of the indicated drug. Proliferation of cisplatin or doxorubicin-treated cells (C) and of cells treated with actinomycin D, RITA or Nutlin-3 (D) was measured after 48 hrs of treatment and displayed as relative survival compared to mock-treated corresponding cells.
### Supplemental Information

#### Q-RT-PCR Primer Sequences

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