FEN1 is Overexpressed in Testis, Lung and Brain Tumors

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Abstract. *Flap endonuclease 1 (FEN1) is a DNA replication/repair protein involved in Okazaki fragment processing, long-patch base excision repair, DNA double-strand break repair and stalled replication fork restart. FEN1 is also important for preservation of telomere stability and thus a key node in maintaining genomic stability. The aim of the present study was to elucidate the level of expression of FEN1 protein in cancer of testis, lung and brain. FEN1 protein expression was studied by Western blot analysis in specimens of tumor tissues compared with the normal tissue from the same patient or normal brain extract. In addition, FEN1 was transiently down-regulated in the glioblastoma cell line LN308 by transfection with siRNA. The transfected cells were treated with cisplatin, temozolomide, nimustine and methyl methanesulfonate (MMS). Induced apoptosis (subG1 fraction) was analysed by flow cytometry. Our data show a clear overexpression of FEN1 in 19/25 samples from testicular tumors (mostly seminomas) and 4/4 samples from lung tumors (non-small cell lung cancer). For brain tumors, 9/11 glioblastoma multiforme and 5/8 astrocytomas expressed FEN1 protein at a higher level than did normal brain tissue. Overall, the data demonstrate that FEN1 overexpression is common in testis, lung and brain tumors. Low-level expression of FEN1 by siRNA down-regulation increased sensitivity to methylating agents (temozolomide, MMS) and cisplatin in LN308 glioma cells, which indicates that altered FEN1 expression might impact the therapeutic response.*

Flap endonuclease-1 (FEN1) is a DNA replication/repair protein with pleiotropic functions. It plays a pivotal role in primer removal during lagging-strand DNA synthesis and Okazaki fragment processing (1) and long-patch base excision repair (BER) by removing the 5'-flaps generated by Polδ/ε during repair synthesis (2). There are reports that FEN1 is also involved in DNA double-strand break (DSB) repair by non-homologous end-joining (3) or homologous recombination (4). FEN1 attenuates DSB generation during DNA replication and suppresses DNA recombination, thus maintaining genomic stability. In addition, in a complex with Werner's syndrome protein it plays an important role in stalled replication fork restart (5). FEN1 depletion leads to telomere dysfunction, phosphorylation of H2AX and sister telomere loss, which shows that FEN1 is important for telomere stability (6, 7).

There is growing evidence that FEN1 expression is related to the development of cancer and the progression of the disease (8). *FEN1* homozygous inactivation in mice leads to early embryonic lethality. Mice with mutations in *FEN1* and the adenomatous polyposis coli (*APC*) genes show increased frequency of adenocarcinomas in the gastrointestinal tract and decreased survival (9, 10). FEN1 is proliferation-dependently regulated (11, 12) and, in mouse fibroblasts, up-regulated in response to genotoxic stress (13). Increased gene expression of *FEN1* was reported in lung cancer cell lines obtained from both small cell lung cancer and non-small cell lung cancer (14). *FEN1* transcripts were also overexpressed in gastric cancer cell lines compared to a normal gastric cell line (15). Since the expression of FEN1 is induced by feeding serum starved adherent cells or following stimulation of lymphocytes with mitogens (11), studies with *in vitro* cultivated cells should be considered with caution. However, *FEN1* up-regulation was also shown in tumors *in situ*. Thus, *FEN1* mRNA level was up-regulated in metastatic prostate cancer (16), pancreatic cancer (17) and neuroblastomas (18). Using immunohistochemistry, FEN1 was shown to be overexpressed in prostate cancer compared to the normal prostate tissue (19). In a recent study, using a cancer profiling array and immunohistochemistry, *FEN1* expression was determined in breast cancer and other tumor entities such as colon, stomach, lung and shown to be overexpressed at the RNA and protein level. It was also found to be expressed at a higher level in samples of metastases. FEN1 overexpression was related to hypomethylation of the promoter (20).

Some reports give evidence that FEN1 might be involved in the acquisition of resistance to anticancer drugs. Myoshi *et al*. isolated a cell line resistant to the farnesyltransferase inhibitor tipifarnib, which is used in the treatment of chronic

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myeloid leukemia (21). By DNA microarray analyses, the authors identified 5 genes with higher expression levels, among them *FEN1*.

In this work, we studied the expression of FEN1 protein by Western blot analysis in testis, lung and brain tumors in order to gain insight into its potential as a diagnostic and therapeutic target. We also studied the effect of transient *FEN1* down-regulation on the sensitivity of LN229 glioblastoma cells to the genotoxin methyl methanesulfonate (MMS) and the anticancer drugs temozolomide (TMZ), nimustine (ACNU) and cisplatin.

Materials and Methods

Preparation of tumor tissue extracts. The collection of testicular and brain tumors was described elsewhere (22, 23). Specimens of surgically removed testicular tumors (n=25) and lung tumors (n=4) as well as the corresponding normal tissue from the same patient were stored in liquid nitrogen. The brain tumor samples [glioblastoma multiforme (n=13) and astrocytoma (n=7)] were compared with commercially available extracts from normal human cerebellum (BioChain, Hayward, CA, USA). For preparation of total tumor tissue extracts, the frozen tissue was crushed in a mortar and transferred to pre-chilled vials, sonicated in 180 μl buffer (20 mM Tris-HCl, pH 8.5; 1 mM EDTA, pH 8; 1 mM β-mercaptoethanol; 5% glycerin; protease-inhibitors: aprotinin 10 μg/ml; bestatin 10 μmol/l; leupeptin 10 μmol/l, pepstatinA 1 μmol/l, PMSF 0.1 mmol/l) and centrifuged at 14 000 rpm at 4˚C for 10 min. The protein concentration was measured by Bradford with Roti-quant® (Roth, Karlsruhe, Germany) at a wavelength of 595 mm.

Western blot analysis. All loading samples were prepared by dilution with bi-distilled water and 4x load buffer (1:3, v/v) to a protein concentration of 1 μg/μl, boiled at 95˚C for 5 min to denature the proteins, then briefly centrifuged and cooled on ice for 5 min. A volume of 30 μl per specimen was loaded onto 4% SDS stacking gel/10% separation gel and run at 40 mA. Thereafter, proteins were blotted onto a nitrocellulose transfer membrane (Protran; Schleicher&Schuell, Dassel, Germany) overnight at 100 mA. Membranes were blocked for 1 h at room temperature in 5% (w/v) fat-free milk powder in phosphate-buffered saline (PBS) containing 0.2% Tween, then incubated overnight at 4˚C with primary mouse anti-FEN1 antibody (1:500; Transduction Laboratories, Lexington, KY, USA), washed 3 times (10 min each) diluted in blocking buffer. After 3 washes in PBS containing 0.2% Tween, the membranes were incubated for 1h with a horse radish peroxidise-coupled secondary antibody (1:3000; Amersham Biosciences AB, Uppsala, Sweden) and blots were developed by a chemiluminescence detection system. All blots were exposed to the same film for 5 min in order to facilitate the comparison between the samples, then stripped, blocked again and incubated with rabbit anti-extracellular signal-regulated kinase2 (ERK2; Santa Cruz Biotechnology, Santa Cruz, CA, USA) used as a loading control, followed by a secondary anti-rabbit antibody. The films were illuminated in an InGenius system and stored by SynGene Snap v.6.05.01 and analysed by the SynGene Tool software v.3.01. (Syngene, Cambridge, UK). The data were plotted as percentage changes in relation to the expression of ERK2. The highest protein level of ERK2 was used to normalize the amount of protein in all samples.

Cell culture of human glioblastoma cells and transient transfection with FEN1 siRNA. The 21mer single-stranded RNA oligonucleotide sequences used were GCAGCACAAUGAUGAGUGCAA (sense) and UUCGUCGUGUUACUACUCACG (antisense) according to (24). They were synthesized at 20 nmol scale by Ambion (Austin, TX, USA) and the RNA strands were resuspended in the supplied nuclease-free water to obtain a final concentration of 100 μM. Annealing of the complementary strands was performed by mixing equal amounts of RNA in the annealing buffer supplied by Ambion followed by heating of the mixture to 90˚C for 1 min and incubation at 37˚C for 1 h. In control experiments, 'scrambled' siRNA (AllStars Negative control siRNA; Qiagen, Hilden, Germany) was used. Glioblastoma cells LN308 were maintained in DMEM/High Glucose (PAA, Pasching, Austria) supplemented with 10% fetal bovine serum (FBS, Biowest, Nuaille, France). The cells were passaged at sub-confluence 4 times and 5×10^4 cells were plated 24 h before transfection in 2.5 ml medium in 6-well plates. Transfections were performed using 40 nM siRNA per well and 2.9 μl Lipofectamine RNAiMAX (Invitrogen, Karlsruhe, Germany) in a total volume of 200 μl, with a complexation time of 30 min. Thereafter, the complexes were diluted to 1 ml and added to cells; after 24 h the transfection mixture was replaced with medium.

Preparation of cell extracts from LN308 cells. Cells were collected by trypsin/EDTA treatment, washed in serum-containing DMEM medium, and once in PBS and the cell pellet was resuspended in a homogenization buffer supplied with COMPLETE Mini Protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), used according to the manufacturer's protocol. The lysate was sonicated six times (40 cycles) on ice using a Branson B15 SONIFIER cell disruptor. The lysate was then spun for 10 min at 14,000×g and the supernatant transferred to a new microcentrifuge tube. Total protein content was determined using Nanodrop ND-1000 spectrophotometer with ND-1000 v.3.0. software (PEQLAB, Erlangen, Germany). Western blot quantification and data analysis was performed as described above.

Treatment with genotoxins. Cells were treated 48 h following siRNA transfection. A 100 mM MMS (Sigma, Munich, Germany) stock solution was prepared by diluting MMS with sterile H_2O. Cells were treated with 0.5 mM MMS for 1 h and the medium was changed. ACNU and cisplatin (both from Sigma) stocks were prepared by dissolving the drugs in sterile H_2O and filtering. The concentration of the ACNU stock solution was 10 mM and of the cisplatin stock solution 1 mg/ml. TMZ (Schering-Plough, Kenilworth, NJ, USA) was dissolved in dimethylsulfoxide (DMSO) and diluted 2-fold in distilled water (stock 35 mM). Stocks were stored at –80˚C. Cells were treated with 50 μM ACNU, 0.2 mM TMZ or 9 μg/ml cisplatin for 6 h, washed once and fresh medium was added.

Determination of apoptosis by flow cytometry. Both adherent and detached cells were collected at certain time-points following treatment and centrifuged at 1,000 rpm for 5 min. Pelleted cells were washed in cold PBS, suspended in 100 μl cold PBS and 2 ml ice-cold 70% ethanol and stored at –20˚C up to 3-4 days. Before analysis, cells were treated with RNase (0.03 mg/ml) and subsequently stained with propidium iodide (16.5 mg/ml) in PBS. Samples were transferred to FACS microtubes and propidium iodide fluorescence was measured by flow cytometry (FACScalibur, Becton Dickinson, Heidelberg, Germany). For each variant, 10,000 cells were counted and the results were expressed as percentage of

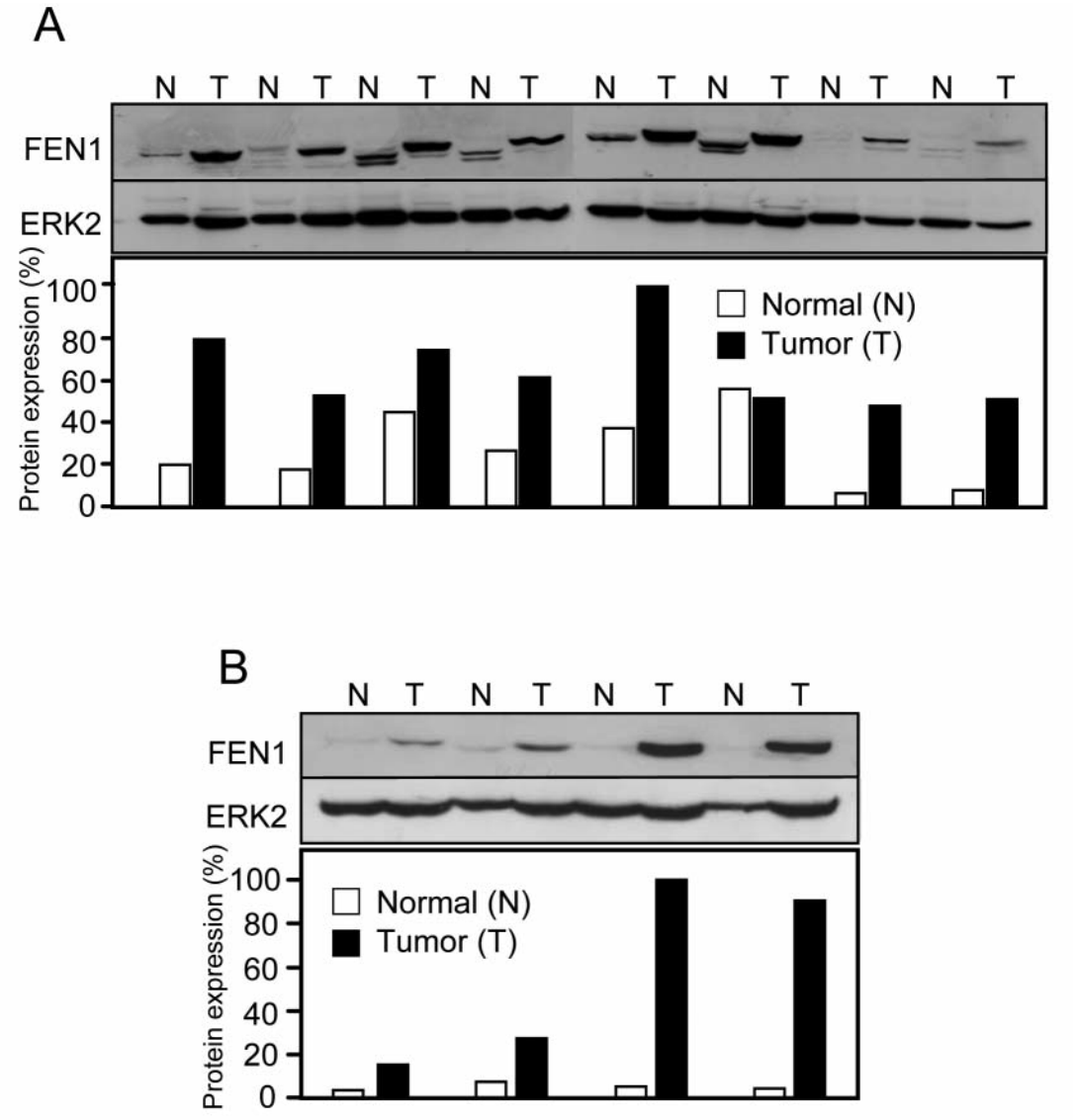

Figure 1. *Protein expression of FEN1 in testis and lung tumors. Specimens of surgically removed seminomas (A) and lung tumors (B) were compared for FEN1 expression to the corresponding normal tissue from the same patient. ERK2 was used as loading control. The upper panel shows a representative Western blot. The lower panel shows the quantification, whereby the highest expressing sample was set to 100%. The quantification was expressed in relation to the loading control (ERK2).*

sub-diploid cells using WinMDI v.2.8 (J. Trotter). All apoptosis experiments were repeated 3 times. Statistical significance was tested by Mann-Whitney non-parametric test in SPSS v.11.0 for Windows™ (LEAD Technologies, Charlotte, NC, USA).

Results

The expression level of FEN1 in testis and lung tumors and the corresponding normal tissue from the same patient is shown in Figure 1. For quantification ERK2 was used as loading control. The data shown in Figure 1A are representative for a set of 8 paired seminoma samples. Among the studied testicular germ cell tumors (n=25), 14/17 samples from seminomas, 2/4 from embryonic carcinomas and 3/4 from mixed tumors displayed overexpression of FEN1 (compared to the corresponding normal tissue of the same patient). For lung cancer, we determined the expression level in 4 paired NSCLC samples and found 4/4 with FEN1 overexpression (Figure 1B). The expression of FEN1 in glioblastoma multiforme (WHO grade IV) and astrocytomas is shown in Figure 2.

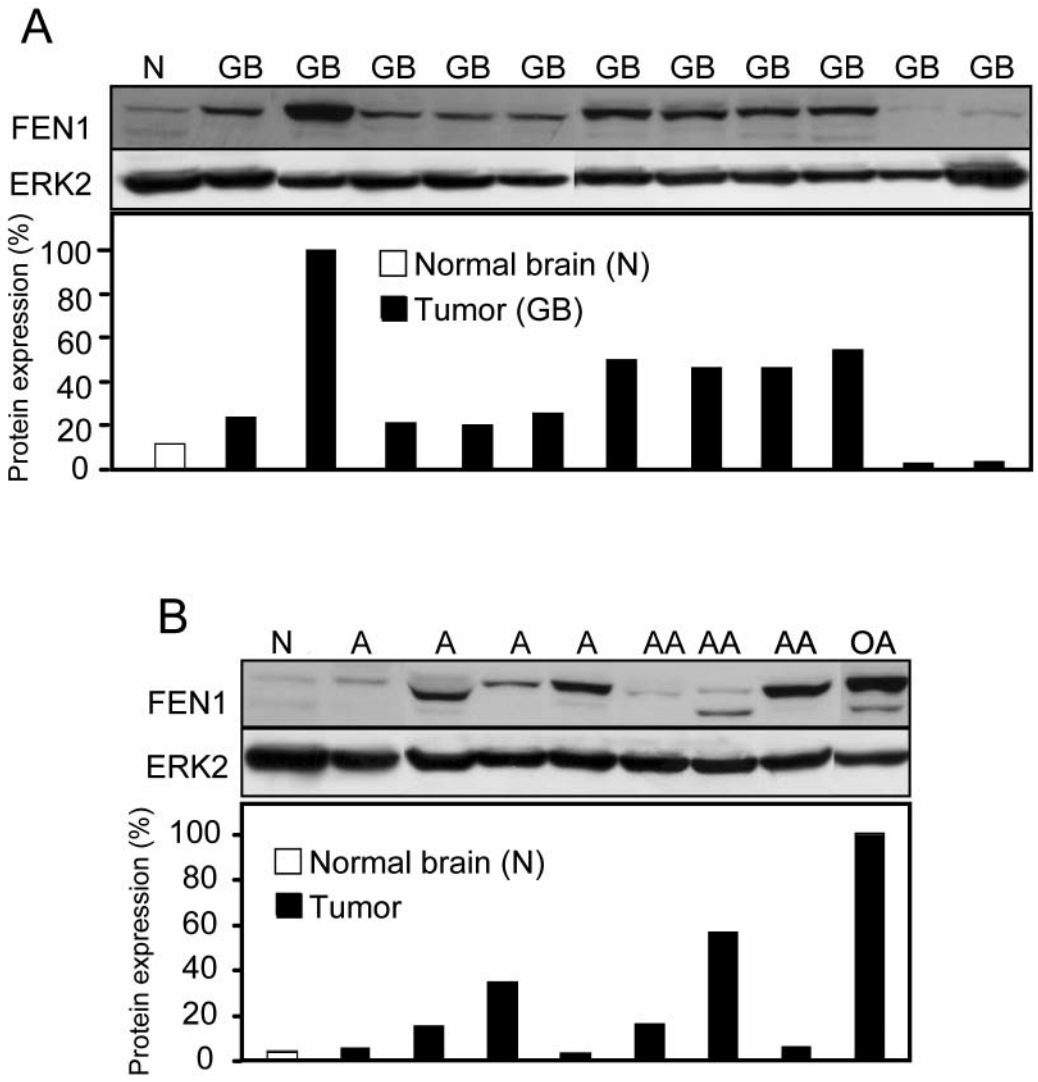

Figure 2. *FEN1 expression in brain tumors. For comparison of glioblastoma (GB) (A) and astrocytoma (B) with normal tissue, human commercially available cerebellum extract (N) was used for control. The upper panel shows a representative Western blot, the lower panel the quantification. The sample with the highest expression level was set to 100%. N, normal human brain; GB, glioblastoma multiforme; A, primary astrocytoma; AA, anaplastic astrocytoma; OA, oligoastrocytoma.*

Since normal brain was not available from the same patient, we compared the expression level in tumors with protein extracts from cerebellum. It can be seen that 9/11 GBM (Figure 2A) and 5/8 astrocytomas (Figure 2B) expressed FEN1 at a higher level than did normal brain. Overall, the data show that FEN1 overexpression is common in testis, lung and brain tumors.

In view of the key function of FEN1 in DNA repair it is pertinent to speculate that the high level of expression in tumors contributes to intrinsic or aquired drug resistance. To gain some insight as to the effect of FEN1 on cell death by anticancer drugs, we down-regulated *FEN1* by siRNA transfection in LN308 glioblastoma cells. *FEN1* down-regulation was confirmed by immunoblotting (Figure 3A). As shown in Figure 3B, FEN1-depleted cells were significantly more sensitive to the methylating agents MMS and TMZ as well as to cisplatin (CDDP). The data indicate that alterations in the FEN1 level may have impact on the tumor cell response to anticancer drugs.

Discussion

To the best of our knowledge, this is the first study showing FEN1 overexpression in glioblastoma and astrocytoma. Overall, the data reported here extend previous observations, indicating that FEN1 at the protein level is up-regulated, not

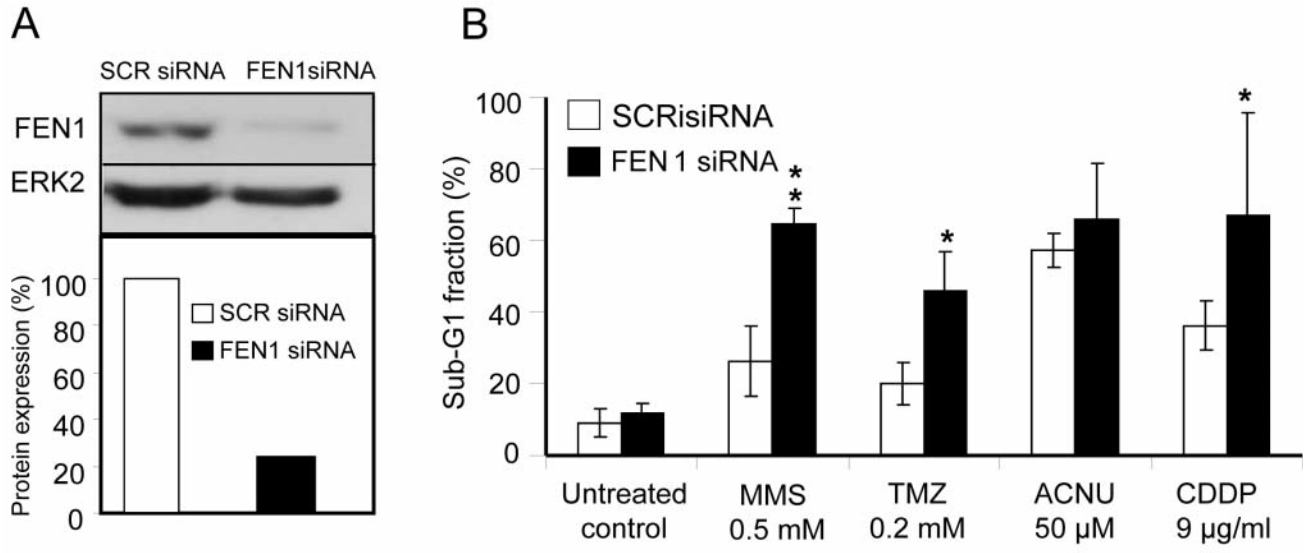

Figure 3. *Transient down-regulation of FEN1 by siRNA in the LN229 glioblastoma cell line provokes sensitivity to methylating agents and cisplatin. A, FEN1 was transiently down-regulated in LN308 cells by transfection with siRNA for FEN1 using Lipofectamine RNAiMAX. For control, a 'scrambled' siRNA (SCRsiRNA) was used for transfection. Total cells extracts were prepared and assayed for FEN1 protein expression by quantitative Western blotting. B, The cells were treated 48 h after transient transfection with either MMS for 1 h, or TMZ, ACNU or CDDP for 6 h at the indicated concentration. After 96 h, cells were harvested, stained and the percentage of sub-G1 fraction was determined by flow cytometry. Data from 3 independent experiments were compared by Mann-Whitney non-parametric test. *p<0.05, **p<0.01, significant increase in apoptosis.*

in all, but in most of the tumors assayed compared to normal tissue. Therefore, it might pose a marker of tumor progression for many types of tumors.

In several studies, FEN1 protein expression was determined by immunohistochemistry, *e.g.* in breast (20) and prostate cancer (19). Immunohistochemistry is not quantitative and possible alterations in the protein cannot be determined. Therefore, we decided to measure the expression level by Western blot analysis. Interestingly, in several samples from testis and brain, a shift in the size of FEN1 protein was observed towards higher molecular weight as compared with the normal tissue (*e.g.* Figure 1A, sample 3, 4 and 6). This might indicate that the protein is posttranslationally modified. There are data that acetylation of FEN1 through the transcriptional coactivator p300 or phosphorylation by cyclin-dependent kinase1-cyclin A leads to reduction of its nuclease and DNA-binding activity *in vitro* and *in vivo* (25, 26).

It has been proposed that increased expression of FEN1 in different types of cancer may reflect the greater proliferation rate of cancer cells compared to normal cells (19) since FEN1 is proliferation-dependent regulated (11) and silenced in terminally differentiated cells (12). Increased FEN1 expression might also be a response to greater DNA damage in cancer cells. We should note that FEN1 is induced at the gene level by genotoxic stress in mouse fibroblasts and that transgenic expression of FEN1 attenuates UV light induced DNA replication inhibition (13). Although the data support the view that FEN1 overexpression protects against genotoxins, it remains to be shown that up-regulation of *FEN1* by genotoxic stress can also occur in human cells, notably in tumor tissue. Irrespective of the mechanism of *FEN1* up-regulation in tumors, it is pertinent to conclude on the basis of available data that FEN1 provides a marker of tumor progression. Whether *FEN1* can become up-regulated in tumors due to radiation and chemotherapy is an intriguing question that remains to be solved.

Since FEN1 is a limiting factor in DNA repair one might speculate that a high expression level in tumors contributes to intrinsic or aquired drug resistance. Therefore, we down-regulated *FEN1* in LN308 glioblastoma cells. FEN1-depleted cells were more sensitive to the methylating agents MMS and TMZ. They were also more sensitive to cisplatin, indicating that the FEN1 level may have impact on the tumor cell response to anticancer drugs. This is in line with reports showing that breast cancer cells with low basal expression of FEN1 are much more sensitive to induction of apoptosis through polyamine depletion compared to breast cancer cells with a higher expression level (27). We should note that *FEN1*-down-regulated cells became sensitive to methylating agents that induce DNA damage repaired by BER, in which FEN1 is decisively involved in the long-patch BER

pathway [for review see (28)]. TMZ is first-line therapy for glioma (WHO III, IV) and, therefore, modulation of FEN1 might have significant impact on chemotherapy by TMZ. FEN1 was also shown to play a role in the final step of nucleotide excision repair (NER) in a complex with ligase I (29). In addition, FEN1 and the NER protein XPG show homology in the DNA-binding domain, which is suggested to confer resistance to the antitumor drug trabectedin (30). Therefore, altered expression of FEN1 might have impact on a broad range of anticancer drugs, including methylating agents and cisplatin. Clearly, further and more detailed studies are required for clarifying the role of FEN1 as a marker of anticancer drug resistance and tumor progression.

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