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ORIGINAL PAPERS

Falkor, a novel cell growth regulator isolated by a functional genetic screen

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A novel cell growth regulator, named Falkor, was identified using a functional approach to mammalian gene cloning, the Genetic Supressor Elements (GSE) method. In this screen, expression of the C-terminal domain of Falkor conferred cells with resistance to cisplatin-induced growth arrest. Expression of the C-terminus of Falkor, but not of the full-length protein, enhanced cell growth both following genotoxic stress and under normal conditions suggesting a general role for this protein in cell growth control. This effect of the C-terminus fragment was abrogated by over-expression of the full-length Falkor, suggesting that the fragment counteracts the function of the full-length protein. Falkor is encoded by a 2-kb mRNA which is present at different levels in various tissues, and is localized in the nucleus of cells. The C-terminal domain of Falkor, isolated from the GSE library, has significant homology to a known human and rat cell growth regulator, SM-20, and to the *C. elegans* protein EGL-9, recently shown to modify the Hypoxia Inducible Factor-1α. The homology suggests that these proteins share a functional domain that is conserved among a family of growth regulation proteins. *Oncogene* (2002) **21,** 6713–6721. doi:10.1038/sj.onc.1205867

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Introduction

The ability of cells to respond to DNA damage is a key property in maintaining genomic stability (Hoeijmakers, 2001). These response pathways include induction of growth arrest, elimination of damaged DNA by induction of the DNA repair machinery, or apoptosis (Amundson *et al*., 1998; Evan and Vousden, 2001). The importance of cellular pathways that prevent the propagation of aberrant cells is underscored by their loss in cancer cells. Inactivation of such growth control pathways allows transformed cells to escape inhibitory growth signals while accumulating genetic alterations. Loss of normal DNA damage response checkpoints is also responsible for the resistance to chemotherapy often occuring in tumor cells.

To identify genes that take part in DNA damage response pathways we used a functional methodology for mammalian gene cloning; the Genetic suppressor elements (GSE) approach (Gudkov *et al*., 1994). This strategy is based on expression of cDNA fragments encoding either peptides that act as inhibitors of protein function or antisense RNA segments which inhibit gene expression. The use of the GSE approach enables the cloning of genes whose inactivation results in a selectable phenotype (Gudkov *et al*., 1994). This method has the advantage over cloning by subtraction or differential display, that it allows the isolation of genes whose function is regulated post-transcriptionally. More importantly, it directly identifies genes that are rate limiting for a biological process.

We applied this method for the isolation of genes critical for cellular DNA-damage response, and we chose Mouse Embryo Fibroblasts (MEFs) as the experimental system because they are primary cells that carry an intact wild type genome, and therefore are assumed to have intact DNA damage response pathways.

Our GSE library was constructed by fragmenting mRNA that was prepared from MEFs treated with the DNA damaging drug cisplatin. The library was delivered to MEFs, that were then treated with cisplatin, and colonies of cells that resisted the cisplatin-induced growth arrest were isolated. In parallel, we have also infected MEFs with a retroviral full-length mouse embryo cDNA library, and carried out the same selection procedure. Using these two libraries, we have isolated two independent clones which overlap and code for the C-terminus of the same protein. The full-length cDNA which codes for a novel protein was cloned, submitted to GenBank and designated Falkor.

Falkor protein is encoded by a 2-Kb mRNA that is expressed at different levels in various tissues, and is localized in the nuclei of cells. Interestingly, the C-terminal domain of Falkor that was isolated from the GSE library and from the mouse embryo cDNA library shares about 70% identity with a known rat and human protein, SM-20 (Dupuy *et al*., 2000; Wax *et al*., 1994). SM-20 was shown by several groups to have a role in cell growth regulation (Lipscomb *et al*., 1999; Madden *et al*., 1996; Wax *et al*., 1996). The C-terminal

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domains of both Falkor and SM-20 are evolutionarily conserved and have strong homology with a known *C. elegans* protein, EGL-9. Recently, EGL-9 and its mammalian homologs, the proline hydroxylases (PHDs), were reported to play an important role in the regulation of the Hypoxia Inducible Factor-1α (HIF-1α) by oxygen level (Bruick and McKnight, 2001; Epstein *et al*., 2001).

We show that expression of a C-terminal fragment of Falkor enhances cell growth under normal conditions. Co-expression of Falkor and its C-terminal fragment abolished the growth enhancement effect of the latter. Falkor is not regulated at the transcriptional level in response to treatment of cells with a DNA-damaging drug.

Taken together, the significant homology of Falkor to SM-20, a known growth regulator and the fact that cells expressing the C-terminal part of Falkor had a growth advantage under DNA damaging conditions as well as under normal conditions, strongly suggest that Falkor is a novel regulator of cell growth.

Results

Isolation of GSEs conferring resistance to cisplatin

The GSE library was generated using mRNA from cisplatin-treated MEFs. In order to construct the MEF GSE library, early passage MEFs were treated with 5 μg/ml cisplatin for 1 h. Poly(A)$^+$RNA was isolated from cells and a normalized GSE library with an average insert size of 500 bp was prepared as described (Gudkov *et al*., 1994) and cloned into the retroviral vector pLXSfi (Figure 1).

Early passage MEFs were infected with library-derived retroviruses produced in Phoenix packaging cells (Pear *et al*., 1993). Infected cells were treated with 2 μg/ml cisplatin for 1 h, which was sufficient to induce an irreversible growth arrest with a typical senescent-like morphology in MEFs. MEFs into which the library has been delivered were selected for continuous growth following treatment with cisplatin. Clones that proliferated on the background of growth arrested cells were collected, and the GSEs conferring resistance to cisplatin-induced growth arrest were isolated by PCR from genomic DNA (Figure 1a). In parallel, the same protocol of selection for cisplatin resistance clones was performed with MEFs infected with the retroviral mouse embryo full-length cDNA library (Clontech).

Cloning of Falkor

Sequencing of the rescued clones revealed that two overlapping fragments of the same cDNA were rescued independently from both the GSE (a 700 bp fragment) and the mouse embryo cDNA libraries (a 1-Kbp fragment).

To verify the phenotype we observed when cells were infected with a whole library, we subcloned the 1-Kb cDNA fragment into the retroviral vector pBabe. Cells were infected with the GSE fragment or with the

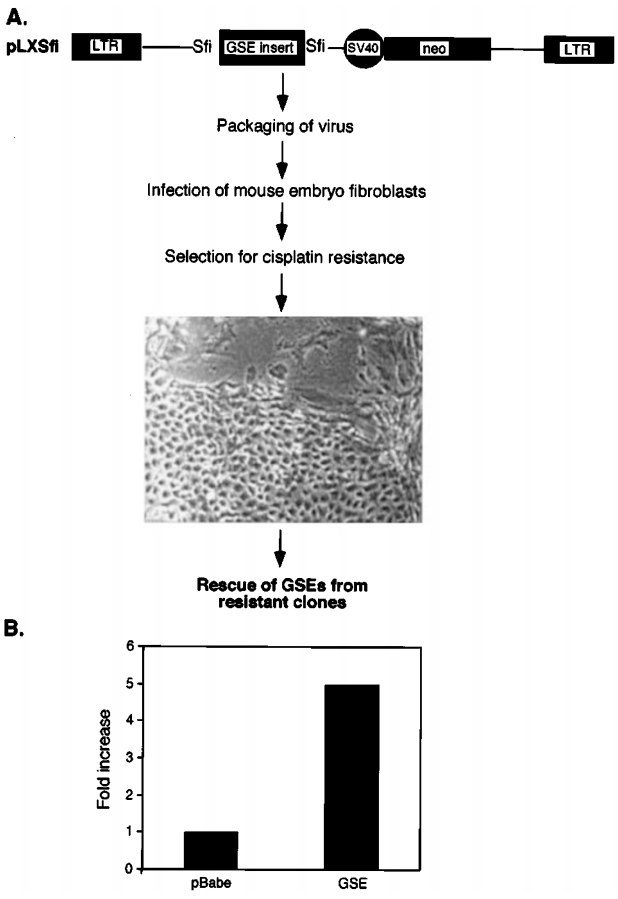

Figure 1 Construction of the MEF-derived GSE library and strategy of functional selection. (**a**) cDNA fragments were generated from mRNA of MEFs treated with cisplatin. The fragments were amplified by PCR and cloned into the Sfi sites of the retroviral vector pLXSfi under the control of the Long Terminal Repeat (LTR) promoter. The resulting GSE library was delivered to MEFs after packaging in Phoenix cells. MEFs were then selected for cisplatin resistance. GSE-DNA was rescued by PCR from genomic DNA of resistant clones. (**b**) Cisplatin resistance conferred by GSE expression. MEFs were infected with the empty vector pBabe-puro or with the GSE cDNA isolated from resistant colonies of library infected cells. Infected cells were treated with 2 μg/ml cisplatin for 1 h. Cells were fixed and stained with crystal violet 14 days later. The experiment was done five times and the figure shows a typical result

empty vector and treated with 2 μg/ml cisplatin for 1 h. We found that the number of resistant colonies in plates of cells infected with the GSE fragment was increased relative to plates of cells infected with the empty vector pBabe. A representative experiment is shown in Figure 1b.

The biologically active GSE/cDNA fragment was sequenced and found to code for the C-terminus of a novel protein. Based on database searches and predictions made from comparison to EST clones, a set of primers suitable for cloning of the full-length cDNA was generated by an Instant RACE at the LabOnWeb site (http://wwww.labonweb.com). Using these primers, a full-length cDNA (Figure 2a) was isolated by RT–PCR from MEF and from the M1/2

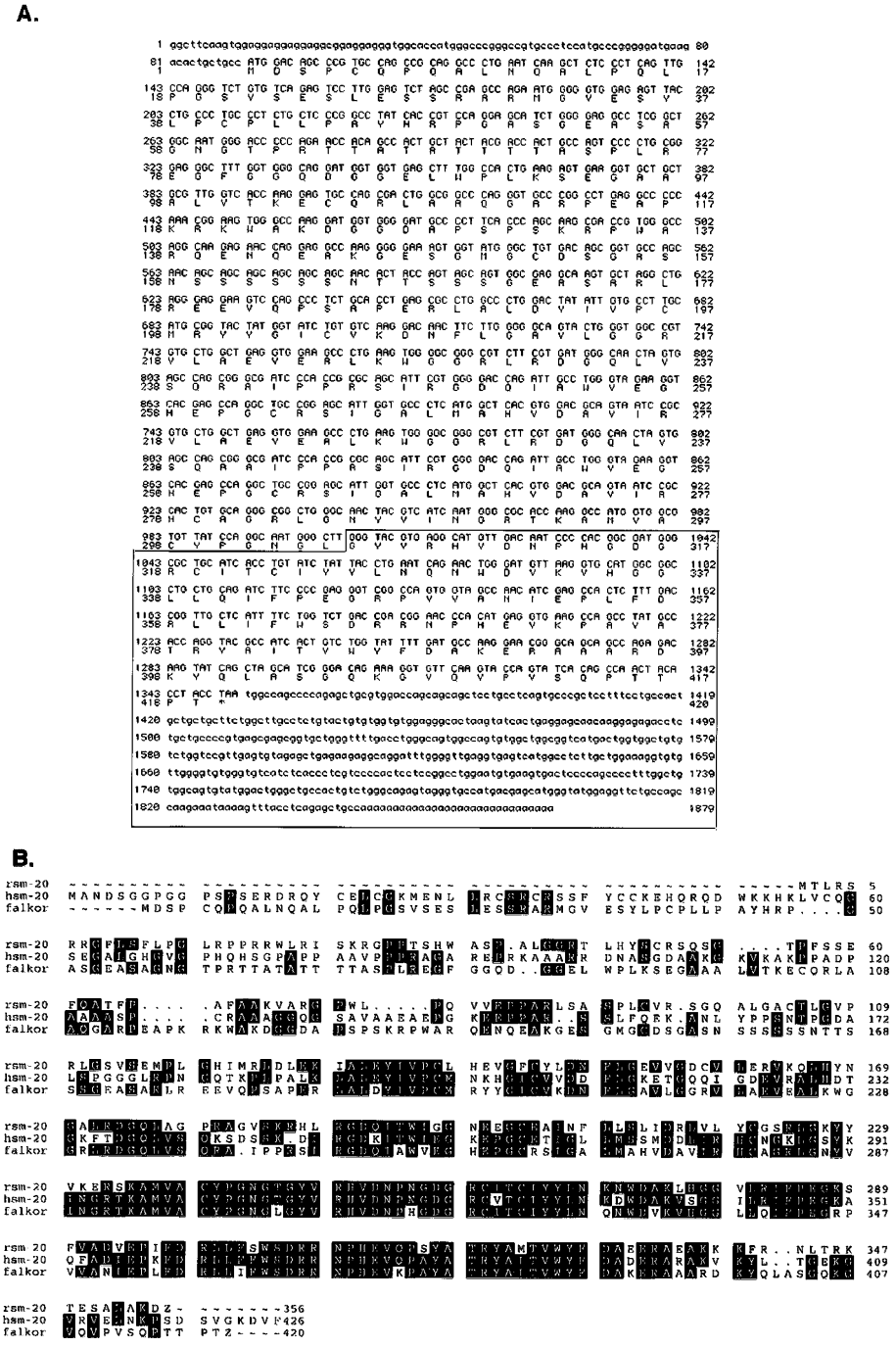

Figure 2 Sequence analysis of Falkor. (**a**) Nucleotide sequence of Falkor derived from mouse cDNA. The C-terminal domain isolated from the mouse embryo and MEF-GSE library is boxed. The 3′UTR contains poly(A)$^{+}$ repeats. (**b**) The amino acid sequence of Falkor was deduced from the longest open reading frame. Homology between Falkor rat SM-20 (rSM-20) and human SM-20 (hSM-20) is shown. Areas of identity are indicated in black. Areas of conservative substitutions are indicated in gray. Sequences for rat and human SM-20 were retrieved from the GenBank. Accession numbers: rat SM-20: NM_019371 human SM-20 AF229245

myeloid cell line mRNA and the novel protein coded by this cDNA was named Falkor.

The size of the deduced full-length mRNA was 2 Kb, consistent with the size of the transcript observed in Northern blots (Figure 3a). Comparison of the isolated cDNA sequence with that of mouse genomic DNA revealed that the Falkor gene contained five exons in a 6.9-kb genomic fragment, with the presumptive initiation codon at exon 1. Comparison of Falkor cDNA sequence to published human genome data revealed a human genomic sequence in chromosome 19q.2 with more than 85% identity to Falkor.

The intron–exon structure of the human genomic sequence was remarkably conserved along the coding region.

Falkor cDNA codes for a 419 amino-acid protein with a predicted size of 46 KD. Search in several protein databases predicted that Falkor includes a number of putative phosphorylation sites for protein kinase C and for casein kinase II. It also contains a putative nuclear localization signal at amino acids 117–120. The presence of a β-barrel jelly roll motif, typical of the 2-oxoglutarate-dependent oxygenase superfamily was reported by Epstein *et al.* (2001).

Comparison of Falkor amino-acid sequence to a protein database showed that this new protein has about 70% identity to a known rat and human protein, SM-20 (Figure 2b). During the course of this study, the human homologs of Falkor, SM-20, and a third family member were cloned by several groups, creating a conserved family of mouse and human proteins (Bruick and McKnight, 2001; Epstein *et al.*, 2001; Seth *et al.*, 2002; Taylor, 2001).

Expression analysis

In order to study the expression pattern of Falkor we performed Northern blot analysis of RNA extracted from different mouse tissues. As seen in Figure 3a, Falkor was expressed at varying levels in all tissues examined. The highest level of expression was detected in testis and the lowest level of expression was found in spleen and in skeletal muscle. Interestingly, Wax *et al.* (1994) showed that SM-20 is not expressed in testis, but is highly expressed in muscle-derived tissues. This implies that despite the similarity between Falkor and SM-20 they have distinct tissue-specific roles in cell growth regulation.

The expression of Falkor was further verified by RT–PCR performed with RNA from different mouse tissues (Figure 3b). Again, varying levels of expression were observed in different tissues, the expression seems to be ubiquitous, and the lowest expression levels was in muscle tissue, consistent with the Northern blot analysis (Figure 3a).

Subcellular localization of Falkor

To examine the subcellular localization of Falkor, we transfected SV-80 cells with a plasmid coding for a fusion protein of Falkor tagged with a Myc epitope at its N-terminus. The fusion protein was efficiently expressed in the transfected cells, and could be readily detected by Western blot analysis (Figure 4a).

SV-80 cells were transiently transfected with the Myc-tagged Falkor. Twenty-four hours after transfection, cells were immunostained with anti-Myc antibodies, or with polyclonal anti-Falkor antibodies, and visualized by fluorescent microscopy.

Immunostaining of SV-80 cells expressing Myc-Falkor showed that Falkor was localized in the nuclei of transfected cells (Figure 4b). This observation is consistent with a predicted Nuclear Localization Signal (NLS) found at amino acids 117–120. Treatment of

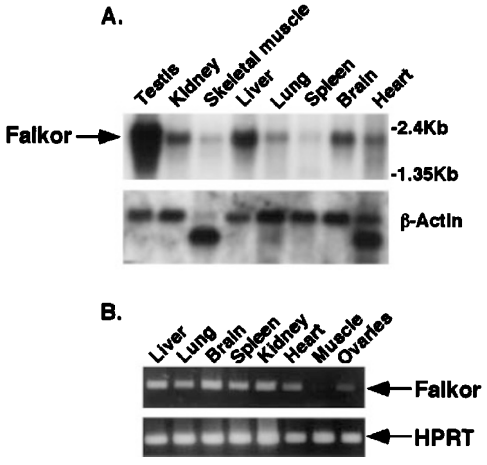

Figure 3 Expression analysis of Falkor. (**a**) Northern blot analysis of mouse tissues with Falkor cDNA probe. A transcript of 2 kb was detected. Testis showed the highest level of expression. The lowest level of expression is detected in spleen and skeletal muscle. The blot was probed with β-actin as a loading control. (**b**) RT–PCR analysis of mouse tissues. Falkor is expressed at all tissues with the lowest level of expression in muscle tissue. RT–PCR with HPRT primers is shown as control

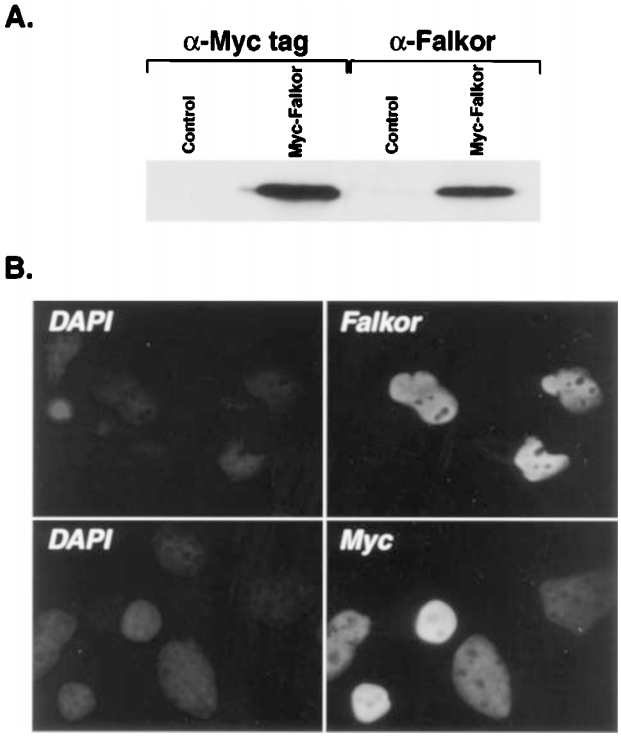

Figure 4 Subcellular localization of Falkor. (**a**) Expression of Myc-Falkor following transient transfection. Myc-Falkor is expressed in transfected, but not in control cells. (**b**) Immunofluorescence analysis of SV-80 cells transiently transfected with Myc-Falkor. Localization of Falkor was determined using an anti-Myc antibody or a polyclonal anti-Falkor antibody, DNA was visualized with DAPI

cells with cisplatin did not change the localization of Falkor (data not shown).

Falkor levels are not changed by cisplatin or by p53

The fact that Falkor was isolated as a rescuing factor from cisplatin-induced growth arrest prompted us to examine the changes in the expression of Falkor in response to cisplatin treatment. Figure 5 shows that there was no change in the level of Falkor mRNA in response to treatment with cisplatin. These results suggest that the putative regulation of Falkor in response to DNA damage is not at the level of expression.

Furthermore, since DNA damage responses are known to be mediated by the activation of p53 (Kastan *et al*., 1991), we examined whether p53 expression affects the expression of Falkor. Comparison of Falkor expression in p53 null cells (HCT116 $-/-$ and MEF $-/-$) with their p53 expressing counterparts (HCT116 $+/+$ and MEFs $+/+$, respectively), indicated no change in the expression of Falkor (Figure 5). The fact that Falkor mRNA levels did not depend on the expression of p53 does not exclude a possible functional link between the two proteins.

Falkor-GSE enhances normal cell growth

We next asked whether the growth promoting effect of the C-terminal domain of Falkor (Falkor-GSE) was restricted to stress conditions, such as those caused by treatment with cisplatin. Passage 3 MEFs were infected with a retrovirus carrying either the full-length Falkor or Falkor-GSE or with the empty vector pBabe-puro. Cells were then selected for 48 h with puromycin. After drug selection, cells were plated at low density (2×10^5 cells/10 cm plate) and their growth rate was followed. As seen in Figure 6a cells infected with Falkor-GSE grew faster and reached a higher density as compared to cells infected with full-length Falkor or with the retroviral vector.

Similar experiments were done in HCT116 cells, a colon carcinoma cell line (Bunz *et al*., 1998). Cells were infected with the retroviral vector pBabe-puro, or with a vector carrying the full-length Falkor or with Falkor-GSE. After selection with Puromycin for 48 h, cells were plated at a very low density in flasks (2×10^4 per 25 cm^2 flask). Ten days later, cells were fixed and stained with crystal violet. Cells infected with Falkor-GSE reached a higher density compared to cells infected with either full-length Falkor or with the vector only. Figure 6b shows crystal violet staining of HCT116 p53 $-/-$ cells. The same experiment was done with the isogenic cell line HCT116 p53 $+/+$, resulting in the same pattern. Thus, the GSE-induced growth seems to be p53-independent.

To verify that the effect on growth induced by the expression of Falkor-GSE was directly related to the endogenous Falkor protein, MEFs infected with Falkor-GSE were next infected with either an empty vector (pBabe-puro) or with the same vector expressing Falkor. After puromycin selection their growth was followed as in Figure 6a. Figure 6c shows that overexpression of Falkor abolished the growth promoting effect of the C-terminal domain. To further demonstrate the functional link between Falkor and the GSE phenotype, MEFs were treated with a Morpholino anti-sense oligonucleotide of Falkor or with a control oligo. To verify delivery, the control oligonucleotide was Fluorescin conjugated and fluorescence was observed under the microscope (not shown). As seen in Figure 6d cells treated with the anti-sense oligonucleotide grew faster and reached a density higher than that of cells treated with the control oligonucleotide.

These results suggest that overexpression of the C-terminal domain of Falkor enhances cell growth under normal conditions and not only after DNA damage, and this effect results from inactivation of the endogenous Falkor protein and thus may indicate a general role for Falkor in cell growth regulation.

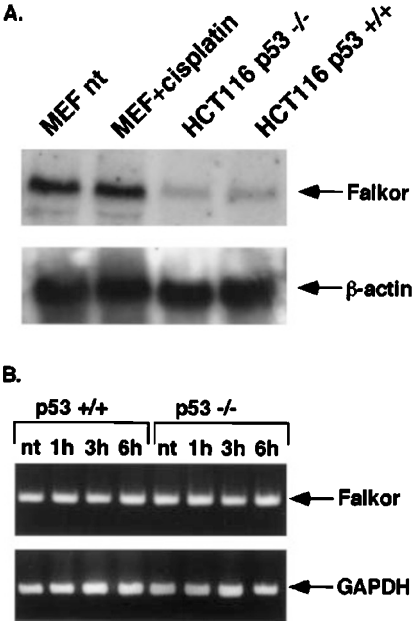

Figure 5 The expression of Falkor is not affected by cisplatin or by p53. (**a**) Northern blot analysis of RNA prepared from MEFs treated with 5 μg/ml cisplatin for 6 h (MEF + cisplatin) or non-treated (MEF nt), and of HCT116 cells expressing p53 (HCT116 p53 $+/+$) or p53 null (HCT116 p53 $-/-$). The blot was probed with β-actin as a loading control. (**b**) RT–PCR of RNA isolated from MEFs treated with 5 μg/ml cisplatin for 1, 3 and 6 h or non-treated (nt). Lanes 1–4 were prepared from p53 $+/+$ MEFs. Lanes 4–8 were prepared from p53 null MEFs (p53 $-/-$). PCR with GAPDH primers was done as control

Discussion

In an effort to identify genes that take part in the response of cells to genotoxic stress, we have used a functional approach to mammalian gene cloning, the GSE method (Gudkov *et al*., 1994). A mouse embryo cDNA library was used in parallel to our own MEF-derived GSE library to select for cDNAs which

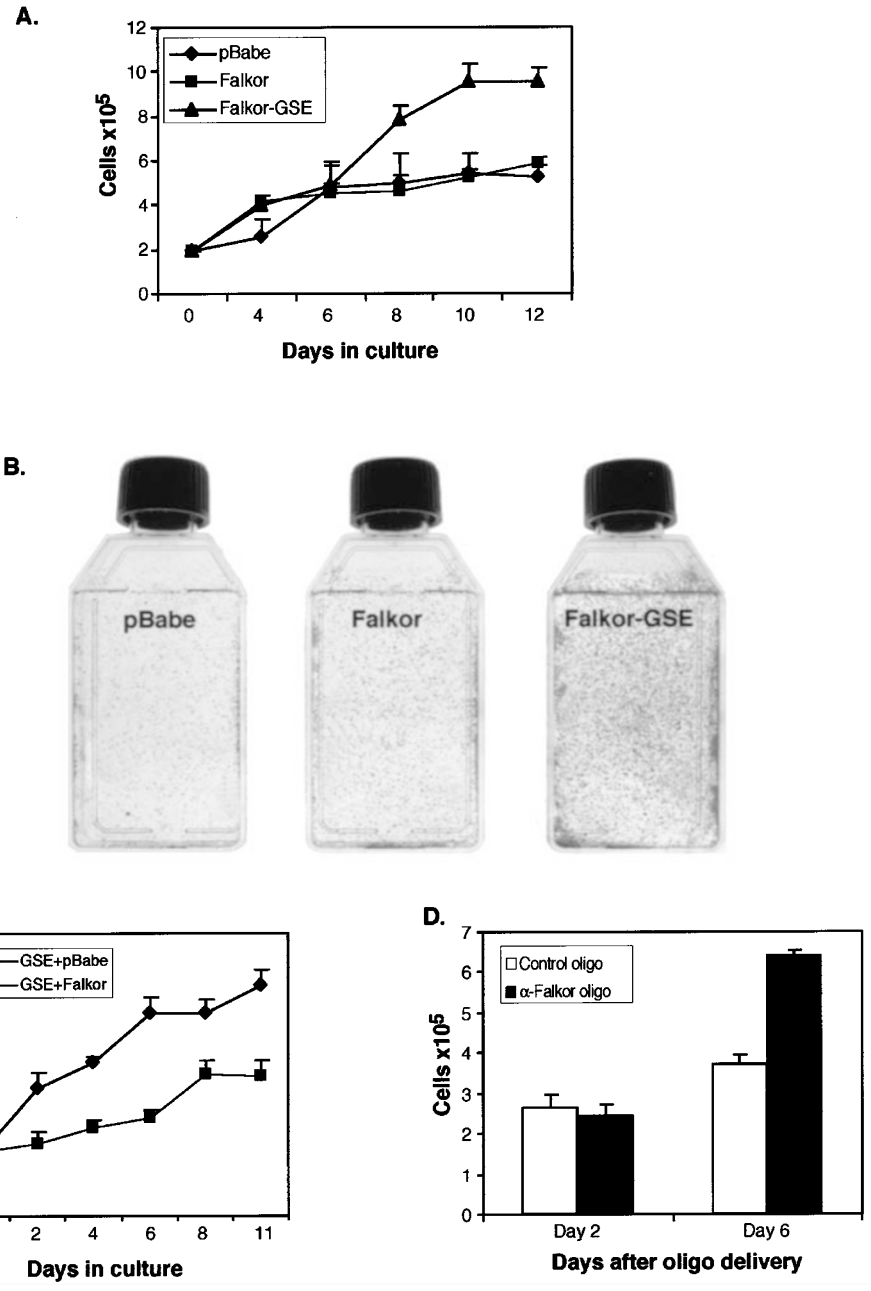

Figure 6 Expression of the C-terminus of Falkor enhances cell growth. (**a**) Passage 3 MEFs infected with retroviruses carrying either an empty vector (pBabe-puro), or full-length Falkor or a C-terminal fragment of Falkor (Falkor-GSE) were plated at 2×10^5/10 cm plate. Cells were harvested and counted every 2 days. The graph represents mean values (+s.d.) from three different plates for every time point at which cells were counted. (**b**) HCT116 cells were infected as in **a** and plated at 2×10^4 cells in a 25 cm^2 flask. Ten days after Puromycin selection cells were fixed and stained with crystal violet. (**c**) Passage 3 MEFs were infected with retroviruses carrying the C-terminal fragment of Falkor (GSE). Twenty-four hours later cells were infected with either empty vector (pBabe-puro) or with the same vector coding for full-length Falkor. After puromycin selection, cells were plated and their growth followed as in (**a**). (**d**) Passage 3 MEFs were delivered with either control oligonucleotide or with anti-Falkor oligonucleotide. The next day, cells were plated at 2×10^5/6 cm plate and their growth was followed for 6 days as in (**a**)

conferred cells with resistance to the growth arrest induced by the DNA damaging drug, cisplatin.

This functional screen resulted in the identification and cloning of a novel gene, Falkor. Falkor was found to be localized in the nuclei of cells. The C-terminus of Falkor was isolated independently from both libraries. When overexpressed in cells, the C-terminal domain of Falkor was found to give cells a growth advantage under normal conditions, and not only following genotoxic stress. This effect seems to result from the dominant negative activity of the C-terminal GSE fragment because it was abolished by the over-expression of Falkor together with its C-terminus. Moreover, cells expressing an anti-sense oligonucleo-

tide specific for Falkor had a growth advantage similar to that observed in cells expressing the GSE fragment of Falkor.

The levels of endogenous Falkor mRNA do not seem to change in cells treated with cisplatin. Moreover, the GSE of Falkor abolished cisplatin-induced growth arrest but the mere over-expression of Falkor was not sufficient to induce growth arrest. These observations suggest that the activity of Falkor is regulated at the post-transcriptional level.

Comparison of Falkor amino-acid sequence to a protein database showed that it has significant homology to two previously described proteins: the rat protein SM-20 and the *C. elegans* protein EGL-9. On the one hand, SM-20 was identified as a growth factor responsive gene in rat smooth muscle cells (Wax *et al*., 1994). On the other hand, it was also reported to be a p53 target gene in rat fibroblasts (Madden *et al*., 1996) and a mitochondrial apoptosis related gene in sympathetic neurons (Lipscomb *et al*., 1999, 2001). These seemingly contradicting reports may suggest that like other cell growth regulators, SM-20 has a distinct regulatory role in different cell types and under different conditions. Interestingly, the C-terminal fragment of Falkor, which we isolated from MEFs resistant to cisplatin-induced growth arrest has the greatest homology to SM-20, while the N-terminal fractions of the two proteins share little similarity with each other (see Figure 2b). Additionally, SM-20 was shown to contain a mitochondrial localization domain at its N-terminus, which is not conserved in Falkor (Lipscomb *et al*., 2001). The finding by Foca *et al*. (2000) reporting increased expression of a cDNA closely related to SM-20 in endometrial carcinomas further supports an important role in growth control for SM-20 and its homologs.

An open reading frame on human chromosome 19 has more than 85% identity with Falkor and most likely represents its human homolog. In parallel to our study, a bioinformatic analysis describing the family of human and mouse homologs of SM-20 was reported. Falkor is identical to the gene termed EGLN2 in this report (Taylor, 2001).

Recently, the *C. elegans* protein EGL-9 was reported to be involved in the regulation of HIF-1α, a transcriptional complex that plays an important role in oxygen homeostasis. EGL-9 and its mammalian homologs, termed PHDs (prolyl hydroxylases) are suggested to function as dioxygenases that regulate HIF-1α by prolyl hydroxylation (Bruick and McKnight, 2001; Epstein *et al*., 2001). Interestingly, the EGL-9 domain that was reported to be the active domain in regulating HIF-1α is conserved in Falkor, which seems to be the mouse ortholog of human PHD1, suggesting that Falkor might have a similar role in mouse cells. In yet another study, PHD1, the human homolog of Falkor was cloned by SAGE as an estrogen-induced gene in a breast cancer cell line and was designated EIT-6 (Seth *et al*., 2002). In this study, EIT-6 was found to promote colony growth in a human breast cancer cell line. These results are seemingly in disagreement with our results which predict a putative growth inhibitory role for Falkor, judged by the anti-sense experiment and the GSE growth promoting effect. This may be explained by different growth effects under different cellular and tissue conditions as was shown for SM-20 and for other known growth regulators which may promote growth or death depending on the cellular context (Jamerson *et al*., 2000; Stanelle *et al*., 2002).

The apparent association of Falkor with the cellular response to cisplatin, implied by our data, and its putative participation in HIF-1α regulation (Epstein *et al*., 2001) may be explained by either different biochemical roles for the mammalian EGL-9 homologs, or by common regulatory pathways activated by both hypoxia and DNA damage.

This hypothesis is supported by a recent study based on sequence profile searches which predicted that EGL-9 and the DNA-repair AlkB are both members of the 2-oxoglutarate dependent dioxygenase superfamily (Aravind and Koonin, 2001). AlkB has an important role in countering toxic DNA modifications caused by alkylating agents (Wei *et al*., 1996). Taken together, we propose that Falkor might be a member of the 2-oxoglutarate dependent dioxygenase superfamily, and that it participates in both the DNA damage and hypoxia responses of cells, which may share a common pathway.

The molecular mechanism that underlies the role of Falkor in growth regulation and in response of cells to DNA damage, and its possible involvement in tumorigenesis, are currently being investigated.

Materials and methods

Preparation of normalized GSE cDNA library

Poly(A)$^+$ RNA was prepared from MEFs that were previously treated with 5 μg/ml cisplatin for 1 h using mRNA purification kit (Pharmacia-Amersham). mRNA was fragmented by boiling for 5 min. The average size of the fragment was 500 bp. Double strand cDNA was synthesized in two steps using the SMART (Switching Mechanism At 5′ end of RNA Transcript) PCR cDNA Library construction kit (Clontech). The primers from this kit add adapters and Sfi cloning sites to the cDNA fragments. Normalization was done as described (Patanjali *et al*., 1991). Briefly, PCR-amplified cDNA was heat-denatured and allowed to reanneal for 24, 48, 72 or 96 h. Single-stranded and double-stranded DNA from each time point was separated by hydroxyapatite chromatography. Each cDNA fraction was reamplified by PCR and analysed by dot-blot hybridizations with different radioactively labeled cDNA probes. The single-strand cDNA fraction obtained after 48 h of reannealing was cloned into the Sfi sites of the retroviral plasmid vector pLXSfi, a modified version of the vector pLXSN. The resulting library contained $\approx 1 \times 10^7$ clones, $>60\%$ of which contained inserts.

Mouse embryo retroviral library

From CLONTECH (Cat. # ML8000BB). Prepared from pooled mRNA of Swiss Webster/NIH embryos. Cloned in the retroviral plasmid pLIB.

Libraries transduction and cisplatin selection

Phoenix-Eco and Phoenix-Ampho packaging cells (purchased from ATCC with permission from Dr Gary Nolan) were transfected with 10 μg DNA of MEF-GSE library or mouse embryo retroviral library (CLONTECH) by a standard calcium phosphate procedure. Culture supernatants were collected 36–48 h post-transfection and filtered. MEFs at passage 3 (7×10^5 per 15 cm plate) were infected with a 1 : 1 mixture of Eco and Ampho filtered viral supernatants in the presence of 4 μg/ml polybrene (Sigma). Fresh viral suspensions were added twice at 6 h intervals. The next day the medium was changed. MEFs were then split 1 : 2 and treated with 2 μg/ml cisplatin for 1 h. The next day 500 μg/ml G418 was added for 5 days to the medium of MEFs infected with MEF-GSE library. pLIB, the retroviral vector in which the CLONTECH library was cloned contains no antibiotic resistance for selection in cells. For cisplatin selection of cells infected with either Falkor-GSE or the empty vector pBabe (Figure 1b), 3×10^5 passage 3 MEFs were plated in 10 cm plates.

GSE DNA extraction and sequencing

Two weeks after cisplatin treatment plates were fixed and stained with crystal violet. Genomic DNA from resistant clones was extracted using QIAmp DNA blood mini kit (QIAGEN). GSE-DNA was retrieved by PCR using primers from LXSfi overlapping the cloning sites (5′LXSfi: GATCCTCCCTTTATCCAGCCC, 3′LXSfi: AAAAGCCTCCTCACTACTTCTGGA). DNA from the mouse embryo library was retrieved using primers from the pLIB vector: 5′LIB: CCTTGAACCTCCTCGTTCG, 3′LIB: TTACTTAAGCTAGCTTGCCAAACCTAC. The PCR products were purified from 1% agarose gel using Gel extraction kit (QIAGEN) and sequenced by Perkin Elmer 3700 DNA analyser. Cloning of full length Falkor was done by RT–PCR using PFU Turbo taq polymerase (Stratagene).

Cell culture

MEFs, SV-80 and Phoenix cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco-BRL) supplemented with 10% fetal calf serum, 2 mM glutamine, and 200 U/ml penicillin and 200 μg/ml streptomycin (Biological Industries, Kibbutz Bet-Haemek, Israel). HCT116 were the generous gift of Dr Bert Vogelstein. HCT116 cells were maintained in McCoy's 5A medium (Biological Industries, Kibbutz Bet-Haemek) supplemented as described for DMEM. MEFs were prepared from BALB/c embryos at embryonic day 14.5.

Northern blot analysis and RT–PCR

Total RNA was prepared from MEFs or HCT116 cells using TriReagent (MRC inc.). RNA was denatured and 30 μg RNA per lane were size fractionated on 1% agarose gel containing 2.2 M formaldehyde and transferred to Hybond N^+ membrane (Amersham).

Mouse multiple tissue Northern blots containing 2 μg of poly(A)$^+$ RNA per lane were purchased from CLONTECH. A 600 bp fragment of Falkor DNA was labelled with α-^{32}P-dCTP using Ready-to-Go DNA labeling kit (Amersham-Pharmacia). Hybridization was performed at 68°C in express hybridization buffer (CLONTECH). The membranes were washed for 40 min at room temperature in 2 × SSC 0.05% SDS and then washed twice in 0.1 × SSC 0.1% SDS at 50°C and autoradiographed. RNA loading on the blots was determined by hybridization with an actin probe.

cDNA for semi-quantitative PCR was prepared using SuperScript II Reverse Transcriptase (Gibco-BRL). Falkor analysis was done using primers 5′-GCAGTACTGGGTGGCCGTGTG and 3′-GTGGTTCCGTCGGTCAGACCA. cDNA loading control was done using GPDH primers.

Protein analysis

Cells were transfected with the expression plasmid for Myc-Falkor fusion protein. Twenty-four hours after transfection cells were lysed with TLB (50 mM Tris pH 7.5, 100 mM NaCl, 1% Triton, 0.1% SDS 1% Na-deoxycholate) and precleared with Protein-A sepharose (Zymed Laboratories Inc). Equal amounts of lysates were incubated O.N. at 4°C with an anti-Myc monoclonal antibody and then incubated while shaking for 2 h with protein-A sepharose at 4°C, and washed three times with TLB. After adding protein loading buffer, samples were boiled for 10 min, separated by SDS–PAGE and blotted onto a nitrocellulose membrane. Membrane was blocked with 5% milk powder in PBST, Myc-Falkor was detected with the anti-Myc monoclonal antibody 9E10, followed by incubation with HRP-conjugated goat-anti-mouse second antibody (Amersham-Pharmacia). Antibodies were visualized by enhanced chemoluminiscence (Amersham-Pharmacia) according to manufacturer's instructions.

Subcellular localization

The open reading frame of Falkor was cloned in pCAN-MycB. PCR-derived constructs were verified by sequencing. The expression constructs were transfected into SV-80 cells using standard calcium phosphate technique. Immunostaining were done 24 h after transfection. Cells were fixed with 4% paraformaldehyde in PBS and then permeabilized (SV-80 cells) with 0.5% Triton X-100. Myc-Falkor was detected with the anti-Myc monoclonal antibody 9E10 followed by the secondary antibody FITC-conjugated $F(ab)_2$ goat anti-mouse (Jackson Laboratories Inc), or by anti-Falkor polyclonal antibody followed by the secondary antibody AlexaFlour-488 conjugated goat anti-rabbit (Molecular Probes). DNA was visualized with DAPI.

Cell growth assay

Passage 3 MEFs were plated at 3×10^5/10 cm plate and infected twice in 6 h intervals with filtered virus containing medium packaged in Phoenix Eco cells. Twenty-four hours later, cells were selected with 1 μg/ml Puromycin for 48 h. For the double infected cells: 24 h after infection with pBabe-GSE, cells were infected with either pBabe-puro or with pBabe-Falkor and then selected with puromycin for 48 h. After puro selection, cells were plated in triplicates at 2×10^5/10 cm plate and counted every 2 days.

Anti-sense oligonucleotide

Passage 3 MEFs were plated at 3×10^5/6 cm plate. The next day, cells were delivered with either a standard control Morpholino Fluorescin-conjugated oligonucleotide or with a specific Falkor anti-sense oligo 5′-CCTGCGGCTGGCACGGGCTGTCCAT-3′ (Gene Tools, LLC). The oligonucleotides were delivered according to the special delivery protocol, based on Ethoxylated Polyethylenimine (EPEI) as

a delivery reagent. Cells were incubated in serum-free medium with a 1:1 mixture of Morpholino/DNA:EPEI special delivery solution. Three hours later, the medium was changed to fresh serum containing medium. The next day, cells were plated at 2×10^5/6 cm plate in triplicates and counted every 2 days.

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Accession numbers
The nucleotide sequence reported in this study was submitted to the GenBank with accession number AF340231.

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