

## Dose-dependent effect of FHIT-inducible expression in Calu-1 lung cancer cell line

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Abnormalities in the expression of the tumour suppressor *fragile histidine triad (FHIT)* gene have been reported in a variety of human tumours, including lung cancer and restoration of its expression in cancer cell lines resulted in the inhibition of proliferation and apoptosis induction. Most of the studies that have assigned a proapoptotic role to the *FHIT* gene were performed in adenoviral-*FHIT*-transduced cancer cells expressing high levels of the Fhit protein. The present work was the first study designed to investigate the effects of *FHIT* gene replacement in a human *FHIT*-negative non-small-cell lung cancer (NSCLC) cell line (Calu-1) by using a hormone-inducible expression system that allows tight modulation of the transgene expression. Through this approach, we demonstrated that a prolonged induction was required to accumulate the Fhit protein at levels adequate to promote a significant decrease of cell proliferation. Analysis of cell-cycle phase distribution showed an accumulation of cells in the G<sub>0</sub>/G<sub>1</sub> phase and a concomitant decrease in the S phase. Moreover, an upregulation of p21<sup>waf1</sup> transcript was found, which could account for the alteration of the cycling properties of the cells. The growth-inhibitory effects observed were not associated with apoptosis appearance, and although in these conditions the Fhit protein content was higher than in normal bronchial human epithelial cells (NHBE), it was still significantly lower than the level capable of inducing apoptosis in Calu-1 cells after adenoviral-mediated *FHIT* gene transfer. These results indicate that the tumour suppressor properties of Fhit are strictly related to its expression level and show that the Fhit protein has a dose-dependent antiproliferative effect on the Fhit-negative Calu-1 lung cancer cell line.

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### Introduction

The short arm of human chromosome 3 is one of the most common sites of chromosomal abnormalities in human malignant diseases. The 3p14.2 region is the focus of particular interest because it contains the tumour suppressor gene *fragile histidine triad (FHIT)* (Ohta *et al.*, 1996).

*FHIT*, a member of the histidine triad family, spans the most active common fragile site in the human genome, FRA3B, and has been reported to undergo genomic alterations resulting in absent or reduced protein expression in a large subset of human tumours (Ishii *et al.*, 2001a). In particular, *FHIT* inactivation seems to be one of the most frequent and earliest events in lung carcinogenesis (Sozzi *et al.*, 1998).

The product of the *FHIT* gene is a 147 amino-acid protein that catalyses the *in vitro* hydrolysis of dinucleoside polyphosphates, with Ap3A as the preferred substrate (Barnes *et al.*, 1996).

Despite numerous reports on the status of the *FHIT* gene in multiple cancers, the biological mechanism of Fhit activity and the cellular pathways associated with its cell-cycle control or tumour suppressor function are not completely understood.

Studies designed to ascertain a possible involvement of *FHIT* in cell growth control and apoptosis included its transfer into cancer cells by using plasmids (Siprashvili *et al.*, 1997; Otterson *et al.*, 1998; Sard *et al.*, 1999; Werner *et al.*, 2000), retroviral (Wu *et al.*, 2000) and adenoviral vectors (Ji *et al.*, 1999; Dumon *et al.*, 2001; Ishii *et al.*, 2001b; Roz *et al.*, 2002). The results obtained have been somewhat conflicting, with some reports showing the ability of Fhit to suppress tumorigenicity of cancer cells and others failing to detect any difference between Fhit re-expressing cells and the parental cell lines (Ishii *et al.*, 2001a). Moreover, the resistance shown in some circumstances by constitutive Fhit re-expressing cells may result from the establishment of a compensatory mechanism, leading to selection for tolerant subpopulations of cells after prolonged expression of the replaced gene. Therefore, in constitutive expression systems, high overexpression of an exogenous tumour suppressor gene might result in a nonphysiological response.

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To avoid the drawbacks rising from the use of constitutive expression systems, we developed an inducible *FHIT* gene expression system to examine the effects of tight modulation of exogenous *FHIT* expression in Calu-1 cells, which lack endogenous Fhit protein. Calu-1 stable transfectants, conditionally expressing the *FHIT* gene, were then analysed for proliferation rate, cell-cycle profile, expression of cell-cycle checkpoint effectors and apoptosis appearance.

## Results

### Inducible expression of *FHIT*

Calu-1 cells were stably transfected with an ecdysone-inducible *Fhit* expression vector. Three clones (2, 4 and 13), which expressed the transgene only upon induction with the ecdysone analogue ponasterone A (Pon A), were selected for subsequent experiments (Figure 1a).

A 16 h treatment of these clones with Pon A activated *FHIT* gene expression and increased the protein levels in a dose-dependent manner (Figure 1b). Analyses were also performed to define the time dependence of Fhit expression: the Fhit protein was already detectable after 4 h of exposure to a Pon A dose of  $7.5 \mu\text{M}$  and its expression level increased progressively with time (Figure 1c).

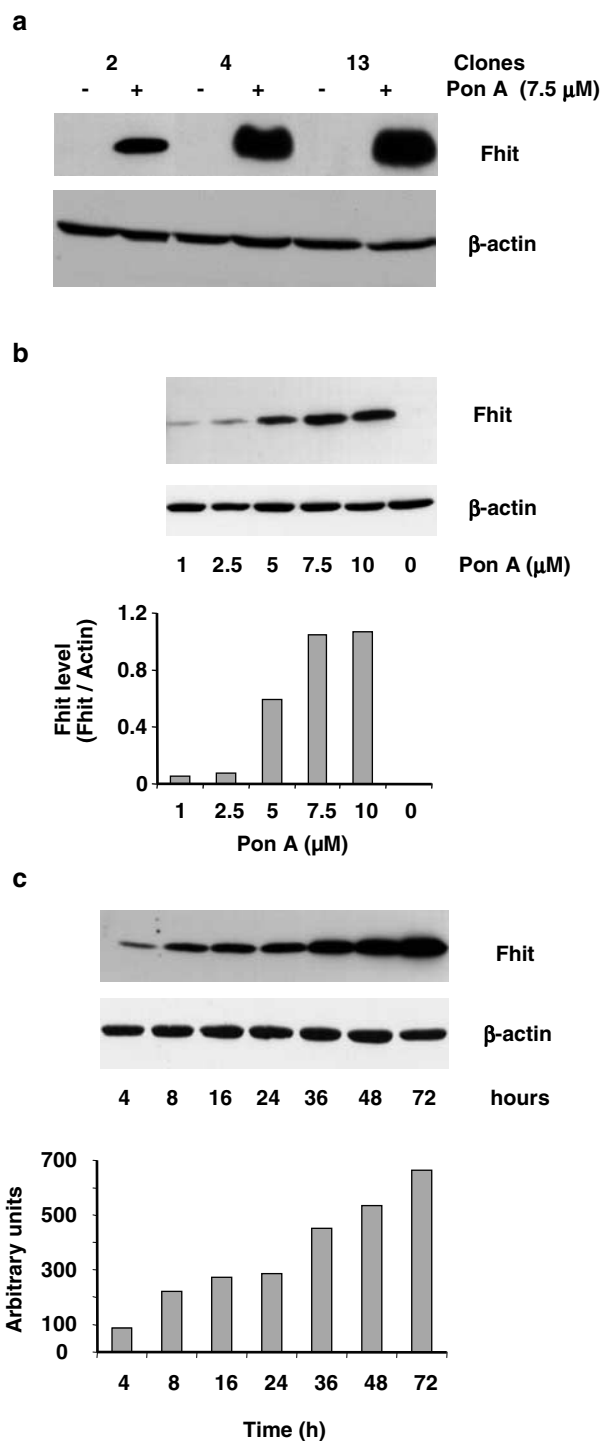
### Fhit protein turnover

Cells were treated with  $7.5 \mu\text{M}$  Pon A for 16 h to activate Fhit expression, then Pon A was removed and the cells were incubated for further 72 h. As shown in Figure 2a, Fhit protein levels gradually decreased but were still detectable even after 72 h. When the cells were treated with  $10 \mu\text{g/ml}$  cycloheximide (CHX) after Pon A removal, the Fhit protein expression level was reduced by 50% after 24 h (Figure 2b), indicating that Fhit is a stable protein, with a half-life of about 24 h.

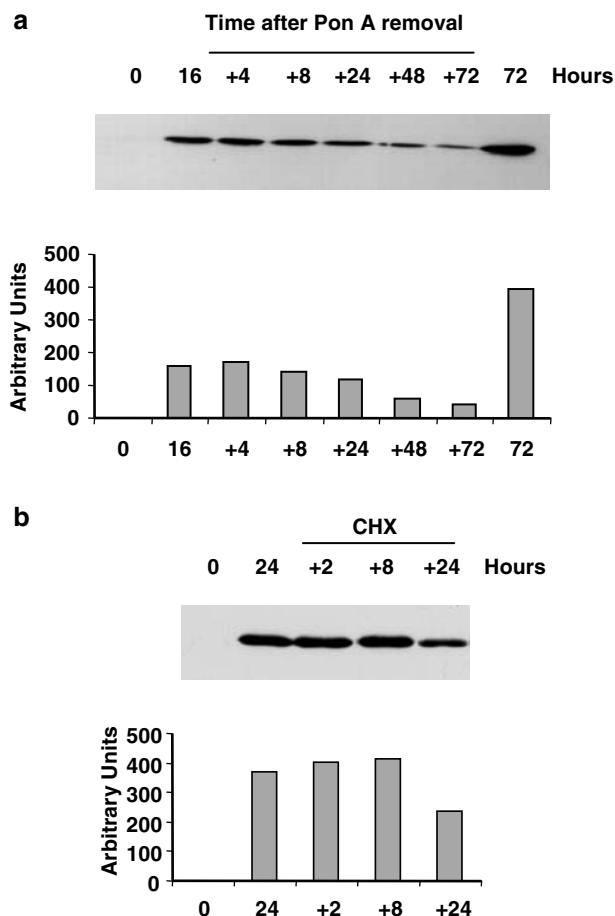
### Effects of Fhit expression on cell proliferation and viability

As assessed by cell counting with trypan blue exclusion (Figure 3a), a decrease of the cell proliferation compared with untreated cells could be observed only after 5–7 days of continuous exposure to a Pon A dose of  $7.5 \mu\text{M}$ , while lower concentrations failed to produce any detectable effect up to 7 days of treatment.

As suggested by Western blot analysis (Figure 3b), the cellular response to *FHIT* gene restoration could be related to a time- and dose-dependent accumulation of the Fhit protein: Fhit levels as high as those induced after 5–7 days of  $7.5 \mu\text{M}$  Pon A treatment were required for the antiproliferative function of the protein, while levels comparable to those expressed by normal human bronchial epithelial cells (NHBE) were not sufficient, at least in this cellular model. A decrease of cell proliferation was also observed in Calu-1 cells from clones 2 and 4 (Figure 4a). In agreement with these data also cell



**Figure 1** Inducible expression of *FHIT* in Calu-1 cell line. (a) A Calu-1 clone constitutively expressing the functional ecdysone receptor was established and then transfected with the pIND-*FHIT* vector to create clones that conditionally express the *FHIT* gene. Cells were cultured in the presence (+) or in the absence (-) of  $7.5 \mu\text{M}$  Pon A for 16 h and then screened for Fhit protein expression. The cell clones 2, 4 and 13, were selected for subsequent experiments. (b) Calu-1 clone 13 cells were incubated in the absence or in the presence of increasing concentration of Pon A for 16 h. (c) Time course of Fhit induction in Calu-1 clone 13 cells after treatment with  $7.5 \mu\text{M}$  Pon A. The level of Fhit protein at each point was quantified by densitometric analysis



**Figure 2** Stability of the inducible Fhit protein. Cells from Calu-1 clone 13 were cultured in the absence or in the presence of  $7.5 \mu\text{M}$  Pon A. **(a)** After 16 h, Pon A was removed and cells were cultured in fresh growth medium. A control sample was prepared by incubating the cells with Pon A for 72 h. **(b)** After 24 h of incubation, Pon A was removed and the cells were then cultured in the presence of CHX ( $10 \mu\text{g/ml}$ ). The level of Fhit protein at each time point was quantified by densitometric analysis

viability, measured by an MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium-bromide) assay, was significantly decreased 6–8 days after *FHIT* gene activation in all three clones (Figure 4b).

#### Effects of Fhit expression on cell-cycle kinetics and apoptosis

Flow cytometric analysis of cell-cycle-phase distribution revealed that Fhit expression caused an increase in the number of cells in  $G_0/G_1$  (60%) with a concomitant decrease in the S phase (26%) compared with the untreated cells ( $G_0/G_1$  49%, S 38%) in Calu-1 clone 13 cells (Figure 5a). Comparable cell-cycle profiles were obtained in cells from clones 2 and 4 (data not shown). To clarify the cause of the  $G_0/G_1$  arrest observed in Calu-1 clones, we examined, by a real-time reverse transcription–polymerase chain reaction (RT–PCR) approach, the mRNA levels of p21<sup>waf1</sup>, previously implicated in the effects of Fhit on the cell cycle (Sard

*et al.*, 1999). A sixfold increase in the expression level of p21<sup>waf1</sup> at 4 days (6.81; range 5.04–9.22) and a threefold increase at 6 days (3.44; range 2.45–4.84) after Pon A treatment was observed (data not shown). Upregulation of the p21<sup>waf1</sup> transcript after Fhit induction was also independently confirmed by Northern blot analysis (Figure 5b). It is worth noting that Calu-1 cells are p53 deleted and therefore the effect of Fhit on p21<sup>waf1</sup> in this experimental system appears to be completely p53 independent.

No apoptotic sub- $G_1$  peak was detected upon Fhit induction, suggesting that growth arrest was not associated with the activation of apoptotic pathways. This result was supported by fluorescence microscopy analysis of Hoechst 33342/propidium iodide-stained cells. Accordingly, Fhit induction by a 9-day Pon A treatment did not lead to the activation of caspase-3 in any of the three clones tested (data not shown), indicating that Fhit expression levels induced in these clones in our experimental conditions were not adequate to promote apoptotic cell death.

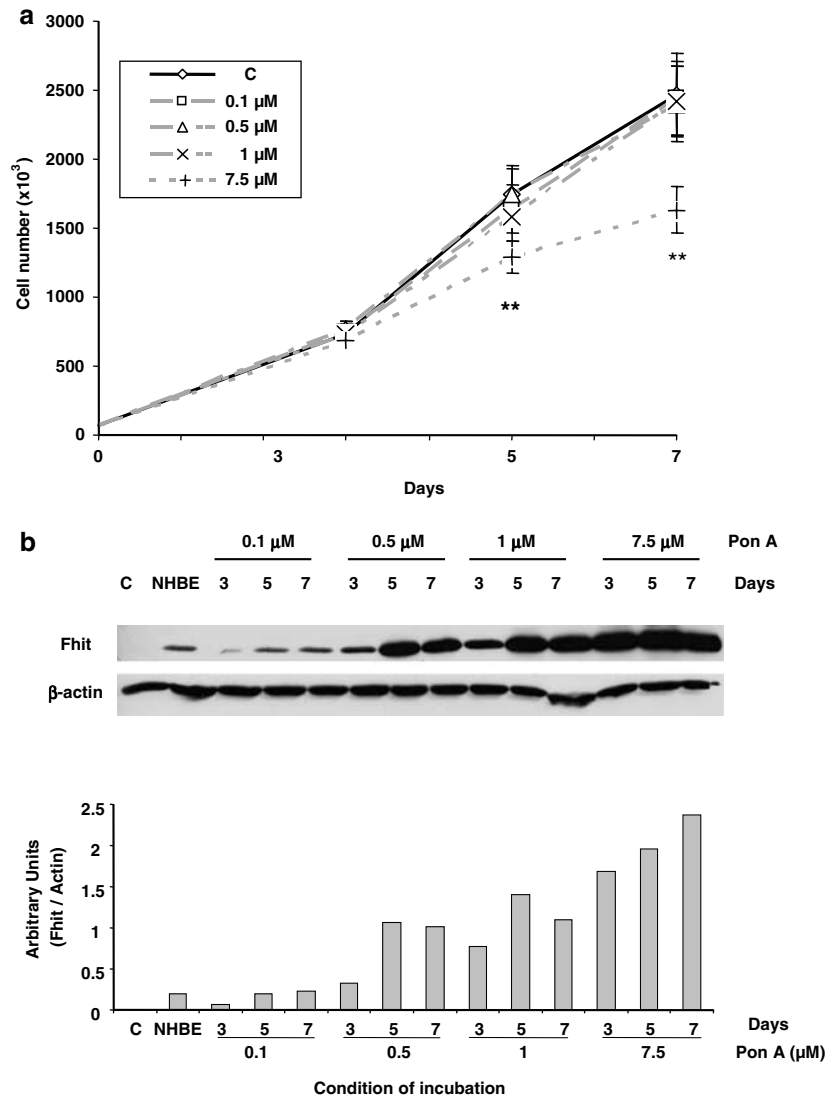
#### Levels of Fhit protein expression in inducible Fhit-expressing Calu-1 transfectants: comparison with adenoviral FHIT-transduced Calu-1 cells and NHBE

In a previous study on lung cancer cell lines (Roz *et al.*, 2002), apoptosis was observed in Calu-1 cells infected with Ad5-Fhit at a multiplicity(ies) of infection (MOI) of 10 (MOI 10; ratio of viral particles to cells during infection). Once established that the Fhit levels in our inducible system might be insufficient to induce apoptosis in the Calu-1 cell line, we compared the expression level of Fhit in the inducible system, in the adenoviral system and in NHBE. Fhit expression levels associated with growth arrest in clone 13 cells were eight times higher than endogenous levels expressed by NHBE cells (Figure 3b), but significantly lower than levels capable of inducing apoptosis in adenoviral-transduced cells (Figure 6a).

The levels of Fhit in the inducible system were also compared with those obtained with the adenoviral system by using increasing MOI of Ad5-Fhit. As shown in Figure 6b, the quantity of protein obtained in Pon A-treated Calu-1 clone 13 cells was comparable with that obtained infecting cells at an MOI of virus between 2 and 5. Interestingly, the analysis of Calu-1 cells infected at low MOIs (0.5, 1, 2) also did not reveal any of the morphological changes associated with apoptosis, while an accumulation of cells in the  $G_0/G_1$  phase of the cell cycle could be detected (data not shown), in accordance to the cell-cycle-phase distribution of Calu-1 clone 13 cells treated with Pon A described above.

#### Discussion

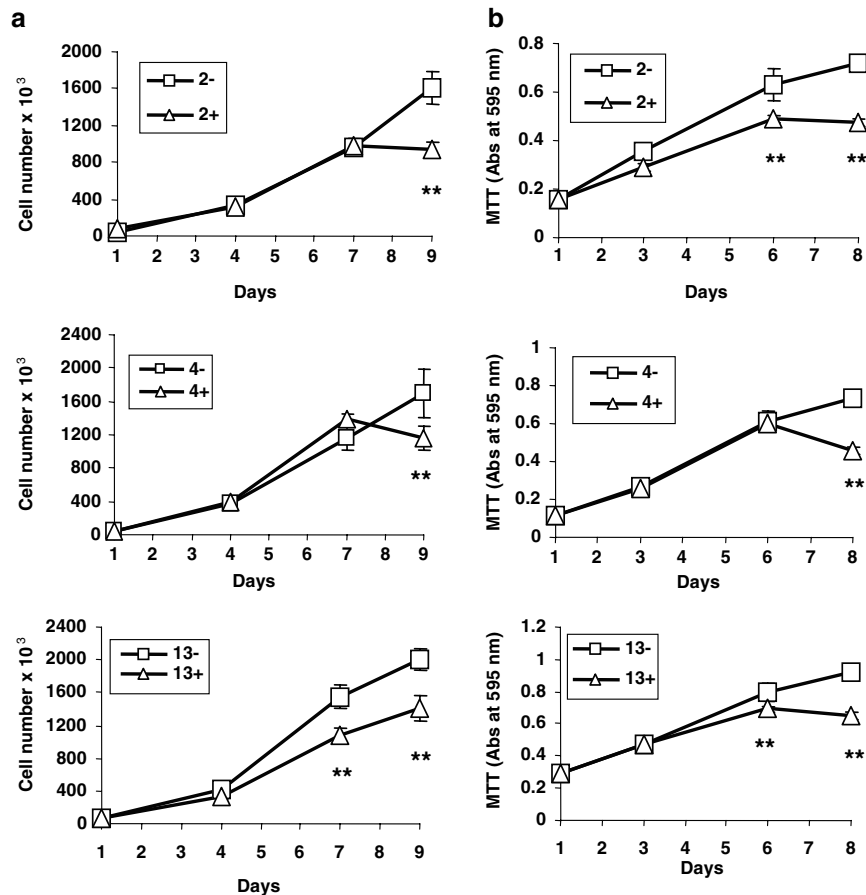
To investigate the role of the Fhit protein in the pathogenesis of epithelial tumours of different histological types, several systems of gene transfer have been employed. Only a small number of stable Fhit



**Figure 3** Analysis of cell proliferation in relation to the level of Fhit induction. Calu-1 clone 13 cells were cultured in fresh medium for 24 h and then incubated in the absence (C) and in the presence of Pon A at different concentrations up to 7 days. **(a)** At the indicated time intervals, cell number was determined by trypan blue exclusion. The mean values ( $\pm$ s.d.) of four independent measurements are given. Differences between induced and not induced conditions:  $**P < 0.01$ . **(b)** Simultaneous Western blot analysis was performed to compare Fhit protein levels. The level of Fhit, normalized to that of  $\beta$ -actin, was quantified by densitometric analysis

transfectants could be isolated using plasmid expression vectors and, although the analysis of their properties gave important insights into Fhit oncosuppressive potential, the onset of compensatory mechanisms that allowed these cells to adapt to grow in culture in the presence of high expression levels of the exogenous *FHIT* transgene, has been reported (Roz *et al.*, 2002). The use of a retroviral vector carrying the *FHIT* transgene has also been described in a study that showed the absence of relevant effects of Fhit expression on tumour cell morphology as well as on *in vitro* and *in vivo* tumorigenicity of cervical and lung cancer cell lines (Wu *et al.*, 2000). On the contrary, the use of an adenoviral vector, Ad5-Fhit, showed high efficiency of infection of a panel of oesophageal, pancreatic, lung and cervical cancer cell lines and resulted in clear signs of

apoptosis, as well as in the onset of a block in the G<sub>1</sub>/S transition of the cell cycle (Ji *et al.*, 1999; Dumon *et al.*, 2001; Ishii *et al.*, 2001b; Roz *et al.*, 2002). Possible explanations for the different results obtained are related to the different sensitivity of the cell lines tested, the expression levels of the transduced gene or the endogenous levels of the Fhit protein. In fact, it has been reported that overexpression of Fhit in normal cells has no effects (Siprashvili *et al.*, 1997; Ji *et al.*, 1999) and some heterogeneity in the endogenous expression levels of this protein in the same cell lines has also been described. Furthermore, studies in the knock-out mouse model have shown an equal incidence of spontaneous and induced tumours in heterozygous Fhit +/– and homozygous Fhit –/– mice, indicating a possible haploinsufficiency of the gene (Zanesi *et al.*, 2001).



**Figure 4** Effect of Pon A treatment on cell proliferation and cell viability in different clones. (a) Cells were incubated in the absence (–) or in the presence (+) of 7.5  $\mu$ M Pon A for 9 days. At the indicated time intervals, cell number was determined by trypan blue exclusion. (b) A total of  $1 \times 10^3$  cells were plated in 96-well microtiter plates and incubated after 24 h in the absence (–) or in the presence (+) of 7.5  $\mu$ M Pon A for 8 days. At the indicated time intervals, cell viability was analysed by MTT assay

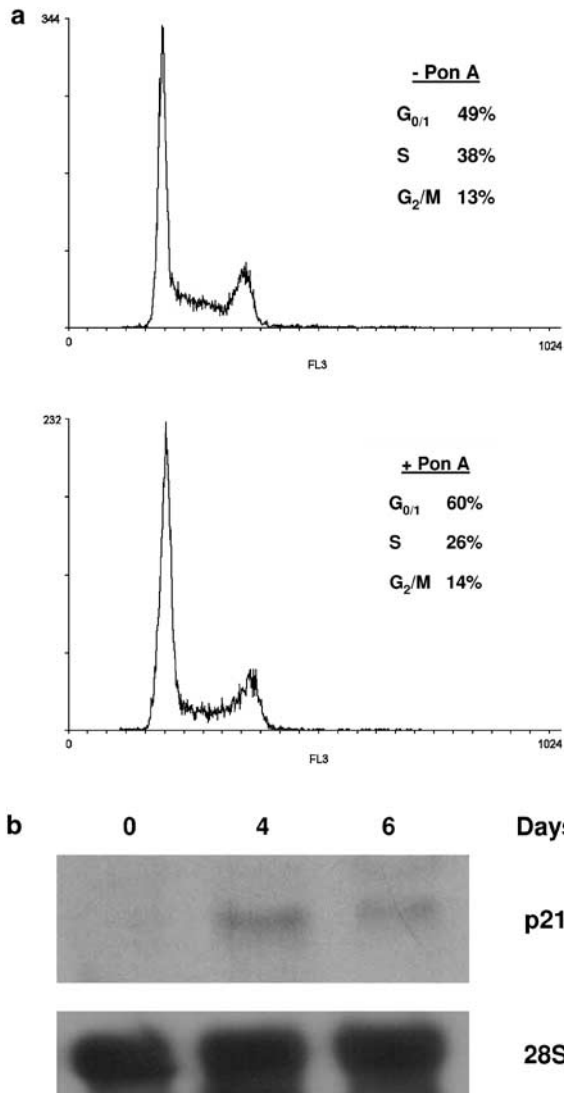
Taken together, these observations strengthen the notion that Fhit-induced effects might be strictly dose dependent and related to the endogenous levels of the protein, and that low or reduced amounts of Fhit protein seem not able to protect completely cells from malignant transformation.

We have collected evidence that FHIT re-expressing cells undergo cell cycle arrest and apoptosis in response to stressful conditions such as those induced by external stimuli (i.e. serum starvation or Fas treatment) better than parental cells lacking Fhit expression (Sard *et al.*, 1999; Roz *et al.*, 2002). In addition, we and others have collected preliminary evidence that also the response to genotoxic damages such as those produced after ionizing radiation, mitomycin C or cisplatin treatment differs in the context of Fhit status in that Fhit-positive cells are more responsive to these stimuli (Fairchild *et al.*, 2000; Roz *et al.*, 2003). Although the precise mechanism of action of Fhit is still unknown, these evidences are consistent with a role as facilitator of the apoptotic response. The observation that loss of Fhit protein expression occurs already in precancerous lesions of the lung starting from mild-severe dysplasia (Sozzi *et al.*, 1998) also suggests that loss of Fhit occur in an early

phase of cancer development probably resulting in resistance to genotoxic stress and apoptosis.

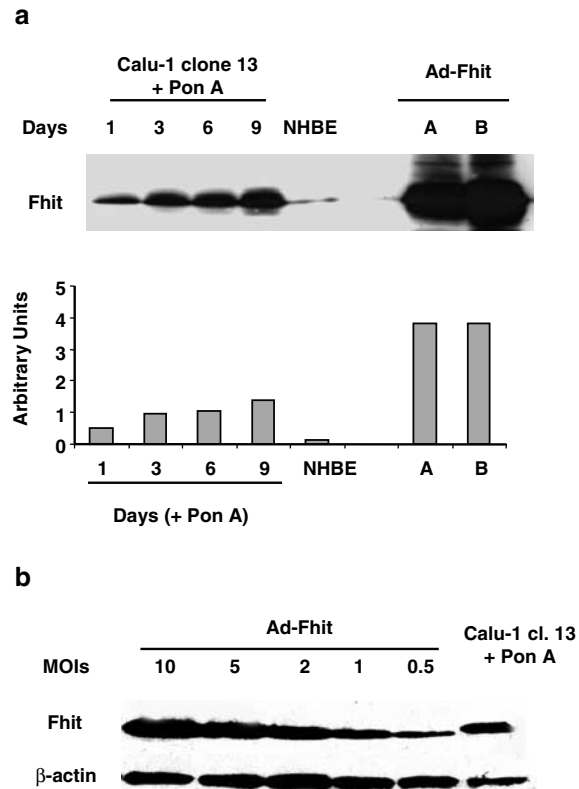
Considering all these evidences and the fact that strong expression of an exogenous transgene might result in a nonphysiological effect, we modified a lung cancer cell line lacking endogenous Fhit expression (Calu-1) in order to obtain conditional expression of the gene by a hormone-inducible mammalian expression system. To our knowledge, this is the first study of Fhit replacement in cancer cells using a plasmid vector with an inducible promoter.

Through this approach we were able to modulate Fhit levels expressed by Calu-1 stable transfectants. We demonstrated that a prolonged induction was required to accumulate Fhit protein at levels capable of inducing a decrease of cell viability and proliferation. Fhit re-expressing clones showed an alteration of the cycling properties with an accumulation of cells in G<sub>0</sub>/G<sub>1</sub> phase and a decrease in S phase. This block seems mediated by p21<sup>waf1</sup> since a clear and rapid increase in p21<sup>waf1</sup> mRNA levels were observed in these cells by real-time RT-PCR, a finding that we already reported in stable Fhit transfectants of the H460 NSCLC cell line (Sard *et al.*, 1999). The growth-inhibitory effects induced by Fhit



**Figure 5** Effects of induced Fhit expression on the cell cycle distribution and on p21<sup>waf1</sup> expression. (a) Cells from Calu-1 clone 13 were cultured in the absence (–) or in the presence (+) of 7.5  $\mu$ M Pon A. After 7 days of culture cells were harvested, reseeded into fresh Pon A-containing or Pon A-free growth medium and incubated for further 2 days. Cells were then stained with propidium iodide and cell-cycle-phase distribution was determined by flow cytometry analysis. (b) Calu-1 clone 13 cells were cultured with 7.5  $\mu$ M Pon A to induce Fhit expression and Northern blot analysis of the p21 transcript was performed on RNA extracted 4 and 6 days after Pon A treatment

expression were not associated with apoptosis appearance, which was instead observed in a variety of lung cancer cell lines, including Calu-1, after adenoviral-mediated *FHIT* gene transfer (Roz *et al.*, 2002). Comparison of the expression levels obtained with the different systems revealed that in the experimental conditions used to induce apoptosis with adenoviral-mediated gene transfer, the level of the protein is two to five times higher than the maximum achievable with the inducible system. Furthermore, it is worth noting that massive apoptosis was observed in Ad-Fhit-infected cells only at 5 days postinfection in serum starvation condition, indicating that reinforced and prolonged Fhit



**Figure 6** Comparison of Fhit levels in the inducible system, in the adenoviral system and in NHBE. (a) Calu-1 clone 13 cells were treated with 7.5  $\mu$ M Pon A to activate *FHIT* gene expression up to 9 days. At the indicated time intervals, cells were extracted and Western blot analysis was performed to compare Fhit protein expression with Fhit levels expressed by NHBE cells and by Ad5-Fhit transduced Calu-1 cells (MOI of 10) incubated in FCS-free medium for 5 days; two infections at the same MOI were performed (A and B). The level of Fhit protein at each point was quantified by densitometric analysis. (b) Calu-1 cells were infected at different MOIs of Ad5-Fhit for 72 h and Calu-1 clone 13 was treated with 7.5  $\mu$ M Pon A for 72 h. The band corresponding to the Fhit protein in Calu-1 Clone 13 cells has a slower migration rate due to the presence of the terminal FLAG peptide

expression is required in order to activate the apoptotic program in association with external apoptotic stimuli.

Although the levels of expression needed to obtain a growth-inhibitory effect are higher than those observed in normal bronchial cells, it has to be considered that established cancer cell lines often harbour many additional genetic changes that may impair the efficacy of the reintroduction of a single gene, especially in highly deregulated pathways such as cell growth and apoptosis. Levels of expression in a differentiated normal tissue might therefore simply represent the quantity of protein expressed under normal conditions and should be used only as starting reference for inferences concerning the levels needed to produce phenotypical effects in highly malignant cancer cell lines. The observation of a precise dose-dependent effect of Fhit re-expression in the present study reinforces previous suggestions that the gene might work as a facilitator in cell cycle control (Sard *et al.*, 1999) and apoptotic pathways (Roz *et al.*, 2002). According to this hypothesis, attainment of a

threshold of expression is needed before the effects can be observed, and we here demonstrate that different thresholds are reached using the hormone-inducible system or the adenoviral-mediated gene transfer resulting in different phenotypical outcomes.

The present data definitely indicate that the tumour suppressor properties of Fhit protein are strictly dependent on its expression level. The effects reported here on proliferation and cell cycle control are also supported by a preliminary analysis of *FHIT*-regulated gene expression patterns using oligonucleotide micro-arrays, performed by comparing Ad-Fhit and Ad-lacZ transduced H460 lung cancer cells, in which most of the genes whose expression was altered at early time points after Fhit transduction were involved in the control of the cell cycle, block of DNA replication and impairment of chromosome function and mitosis (Roz *et al.*, 2004).

In conclusion, our data support *FHIT* involvement in the regulation of critical cellular responses, consistent with its tumour suppressor function. The use of conditional *FHIT* gene expression system will be a useful tool for studying the effects of the Fhit protein under controlled experimental conditions.

## Materials and methods

### Cell culture

The human NSCLC cell line Calu-1 was purchased from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in RPMI 1640 containing 10% heat-inactivated fetal calf serum, at 37°C in a water-saturated atmosphere of 5% CO<sub>2</sub> in air. NHBE were obtained from Clonetics (Walkersville, MD, USA) and cultured as recommended. Low passage 293 cells for adenovirus production were purchased from Microbix Biosystems Inc. (Toronto, Ontario, Canada) and maintained in EMEM with 5% heat-inactivated horse serum, 2 mM glutamine and 1% penicillin and streptomycin.

### FHIT gene-inducible expression system

The ecdysone-inducible mammalian expression system (Invitrogen, Carlsbad, CA, USA) was used to generate cell lines that conditionally express Fhit. The system uses the steroid hormone ecdysone analogue Pon A to activate the expression of the gene via a heterodimeric nuclear receptor. A human full-length *FHIT* cDNA with a FLAG octapeptide sequence was ligated into the *Hind*III and *Apa*I sites of the pIND vector. Calu-1 cells ( $3 \times 10^6$ ) were transfected by a standard electroporation procedure, as described previously (Fumarola *et al.*, 2001). Cells were first transfected with 10 µg of pVgRXXR vector, which contains the receptor subunits, and plated in growth medium with 50–200 µg/ml of zeocin antibiotic. In all, 30 selected clones were then tested for Pon A-inducible gene expression by transiently transfecting with a reported plasmid expressing β-galactosidase. Cells from the clone showing the higher induced signal were transfected with 10 µg of pIND-*FHIT* vector and cultured in the presence of 50 µg/ml zeocin and 400–700 µg/ml G418 (geneticin) to generate stable clones conditionally expressing the *FHIT* gene from the Pon A-inducible promoter.

In total, 36 selected clones from zeocin/G418-resistant culture were tested for inducible Fhit expression after a 16 h

treatment with 7.5 µM Pon A. Zeocin, G418 and Pon A were purchased from Invitrogen.

### Western blot analysis

Procedures for protein extraction, solubilization and protein analysis by 1-D PAGE were previously described in detail (Petronini *et al.*, 1993). Protein (50–100 µg) from lysates were resolved by 5–15% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were incubated with a rabbit anti-human Fhit polyclonal antibody at 1:500 dilution (Zymed, San Francisco, CA, USA) and a horseradish peroxidase (HRP)-conjugated secondary antibody at 1:20 000 dilution (Amersham Pharmacia Biotech, Buckinghamshire, UK). Immunoreactive bands were visualized by using an enhanced chemiluminescence system (SuperSignal West Pico, Pierce, Rockford, IL, USA).

### Determination of cell growth and survival

Proliferation rate and cell survival were determined by: (a) cell counting: cells were detached from the plates by trypsinization and counted in a Bürker hemocytometer by trypan blue exclusion; (b) an MTT assay, based on the viability of live cells to utilize thiazol blue and its subsequent conversion into a dark blue formazan (Petronini *et al.*, 1996).

### Cell cycle and Northern blot analysis

Distribution of the cells in the cell cycle was determined by propidium iodide staining and flow cytometry analysis. Briefly,  $5 \times 10^5$  cells were harvested, washed in PBS and incubated overnight at 4°C in 1 ml of hypotonic fluorochrome solution, containing 50 µg/ml propidium iodide, 0.1% sodium citrate and 0.1% Triton X-100. All reagents were obtained from Sigma-Aldrich (St Louis, MO, USA). Analysis was performed with a Coulter EPICS XL-MCL cytometer (Coulter Co., Miami, FL, USA). Cell-cycle distributions were analysed by MultiCycle DNA Content and Cell Cycle Analysis Software (Phoenix Flow Systems, Inc., San Diego, CA, USA). Northern blot analysis of p21<sup>waf1</sup> was performed using standard techniques as described previously (Sard *et al.*, 1999).

### Detection of apoptosis

Cell apoptosis was assessed both morphologically and in terms of increased caspase-3 activity as described previously (Fumarola *et al.*, 2001; Alfieri *et al.*, 2002).

### Adenoviral-mediated gene transfer

The preparation of recombinant, E1 and E3 deleted, adenoviral vectors expressing the Fhit protein or the control proteins lacZ and GFP was performed using standard techniques as described earlier (Roz *et al.*, 2002). For transduction experiments, Calu-1 cells were seeded into six-well plates ( $3 \times 10^5$  cells) or 10 mm Petri dishes ( $1 \times 10^6$  cells) and allowed to adhere. Virus was then added at the required MOI in a small amount of serum-free medium and cells were kept for at least 2 h before adding complete medium. Transgene expression after infection was routinely confirmed by Western blot analysis.

### Quantitative real-time RT-PCR

To evaluate the expression level of p21<sup>waf1</sup> mRNA in clone 13 Calu-1 cells treated with Pon A, quantitative real-time

RT-PCR was performed. Total RNA (1 µg) from cells untreated and treated with Pon A for 4 and 6 h was reverse transcribed into cDNA using ImProm-II Reverse Transcription System (Promega). Each RNA preparation was previously treated with RNase free DNase (Ambion) to eliminate genomic DNA contamination. cDNAs were diluted 10 times and 1 µl of the diluted samples was used in the PCR reaction performed with the ready to use p21<sup>waf1</sup> Assay-on-Demand™ by Applied Biosystems and the experiment was run on a Gene Amp 5700 Sequence Detection System (Applied Biosystems). Two endogenous controls (HPRT and TBP) were used as references to normalize the different samples and for relative quantization of gene expression; the data were analysed by the comparative Ct method ( $\Delta\Delta Ct$ ).

#### Statistical analysis

The data shown are mean values of at least three different experiments and expressed as mean  $\pm$  s.d. Student's *t*-test was

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used to compare data. A *P*-value of less than 0.05 or 0.01 was considered as statistically significant.

#### Abbreviations

FHIT, fragile histidine triad; NSCLC, non-small-cell lung cancer; NHBE, normal human bronchial epithelial cells; Pon A, ponasterone A; HRP, horseradish peroxidase; RT-PCR, reverse transcription-polymerase chain reaction; MOI(s), multiplicity(ies) of infection; CHX, cycloheximide.

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