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Inducible Activation of IFI 16 Results in Suppression of Telomerase Activity, Growth Suppression and Induction of Cellular Senescence

Christopher J.P. Clarke,[1,2] Linda L. Hii,[1,2] Jessica E. Bolden,[1,3] and Ricky W. Johnstone[1,2]*

[1]*Cancer Immunology Program, Peter MacCallum Cancer Centre, East Melbourne, Victoria 3002, Australia*

[2]*Department of Pathology, University of Melbourne, Parkville, Victoria 3045, Australia*

[3]*Department of Biochemistry, University of Melbourne, Parkville, Victoria 3045, Australia*

ABSTRACT

Expression of the human HIN-200 family member IFI 16 has been reported to suppress cell growth and contribute to the onset of cellular senescence. However the molecular events involved in this process have not been fully characterised. We fused IFI 16 to the estrogen receptor ligand-binding domain to establish an inducible model for studying the molecular events that cause these phenomena. In cells induced to express the ER-IFI 16 within the nucleus there was a decrease in cellular proliferation and concomitant growth arrest in the G1 phase of the cell cycle. Unlike previous reports, this did not appear to involve the p53-p21$^{WAF1/CIP1}$-cdk2-pRb pathway. Following nuclear expression of ER-IFI 16 we noted senescence-like morphological changes and expression of senescence-associated β-galactosidase in growth arrested cells. Importantly, we also found a marked reduction in telomerase activity in arrested cells compared to controls. Moreover, IFI 16 and hTERT co-localised within the nucleus and these two proteins physically interacted in vivo and in vitro. Together, these data suggest that IFI 16 may act as an endogenous regulator of telomerase activity and, through its interaction with hTERT, contributes to the inhibition of proliferation and induces a senescence-like state. J. Cell. Biochem. 109: 103–112, 2010.

KEY WORDS: TELOMERASE; IFI 16; SENESCENCE; INTERFERON; HIN-200

IFI 16 is a member of the HIN-200 family of interferon-inducible genes that can inhibit cell proliferation and are putative tumour suppressor proteins [Ludlow et al., 2005]. HIN-200 proteins can sensitise cells to apoptosis [Wen et al., 2000; Ding et al., 2002], but more commonly trigger cell cycle arrest at the transition from G1 to S phase [Lembo et al., 1995, 1998]. This is thought to be because HIN-200 proteins can target pathways involving p53, p21$^{WAF1/CIP1}$, cdk2 and pRb leading to repression of E2F transcriptional targets [Asefa et al., 2004].

The possibility that IFI 16 is associated with differentiated or senescent phenotypes has also been studied. Increased expression of IFI 16 correlated with passage number, senescent morphology and expression of senescence-associated β-galactosidase in primary prostate epithelium [Xin et al., 2003]. In addition, knockdown of wild type IFI 16 in human dermal fibroblasts prevented the cell cycle arrest and the associated senescence-like phenotype [Xin et al., 2004]. Immortalised cells avoid senescence by maintaining telomere length, most commonly through telomerase activity [Kim et al., 1994]. This enzyme consists of protein sub-unit, hTERT that contains enzymic activity and an RNA sub-unit (hTR) that contains the template for telomere ends [Weinrich et al., 1997]. Cells can be forced into senescence by inhibiting hTERT, but usually after weeks or months depending upon initial telomere length and the rate of telomere attrition [Damm et al., 2001]. However, it is also possible to induce apoptosis by interfering with telomere function which occurs more rapidly via a DNA damage response [Folini et al., 2007]. Interestingly, depleting hTR by RNA interference causes a rapid growth arrest in a period of days [Li et al., 2005].

We investigated the effect of the human HIN-200 family protein IFI 16 on cell proliferation. When expressed as an estrogen receptor (ER) ligand-binding domain fusion protein IFI 16 was sequestered in the cytoplasm, but conditionally translocated to the nucleus in cells treated with the estrogen receptor antagonist 4-hydroxytamoxifen (4OHT). Translocation of the ER-IFI 16 fusion protein to the nucleus inhibited cellular proliferation by triggering arrest in the G1 phase of cell cycle, but did not initiate DNA fragmentation. In contrast to

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: Ricky Johnstone, Cancer Immunology Program, Peter MacCallum Cancer Centre, St Andrews Place, East Melbourne, Victoria 3002, Australia. E-mail: ricky.johnstone@petermac.org

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previous studies [Kwak et al., 2003; Ding et al., 2004; Xin et al., 2004], no change in expression and/or phosphorylation of p53, $p21^{WAF1/CIP1}$ Cdk2 or pRb was observed, indicating that this molecular pathway was not involved. However, there was a marked increase in cells with a flattened morphology expressing 'senescence-associated' β-galactosidase. We observed that ER-IFI 16 co-localised with hTERT in 4OHT-treated cells and measured a decrease in telomerase activity following nuclear expression of ER-IFI 16. IFI 16 partially co-localised with IFI 16 and interacted with hTERT in vitro. We propose that the effect of IFI 16 on cell growth is linked to its ability to bind and functionally inactivate hTERT leading to induction of a senescent-like state.

MATERIALS AND METHODS

CELL LINES, TISSUE CULTURE AND ANTIBODIES

MCF-7 cells (ATCC) were cultured in RPMI, 2mM Glutamine (JRH, Parkville, Australia), and 10% FCS (ThermoTrace, Melbourne, Australia) and used to derive clones expressing a mutated estrogen receptor [Littlewood et al., 1995] fused at the N-terminus of IFI 16B. Mouse mAb to IFI 16 has been previously described [Dawson and Trapani, 1995] and rabbit pAb to IFI 16 was raised in rabbits immunised with recombinant GST fused to the N-terminal fragment of IFI 16 (amino acids 1-159). Anti-hTERT (sc-7215), $p21^{waf}$ (F5) cyclin-dependent kinase 2 (M2), p53 (DO1) pRb (1F8) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The specificity of SC-7215 was confirmed by Western blot (Fig. S1)

PLASMIDS AND TRANSFECTIONS

Transfections were performed using Fugene 6 (Roche Diagnostics Australia Pty., Castle Hill, Australia) according to the manufacturer's instructions. The coding regions of IFI 16A B and C isoforms were cloned into pcDNA3. pcDNA3 ER-IFI16 was generated by PCR-amplifying the mutated estrogen receptor from pBabepuroEKLF-ER [Coghill et al., 2001] and cloning into pcDNA3 upstream and in frame of IFI 16B. pCI hTERT and pCIneo were kind gifts from Prof Roger Reddel (CMRI, Sydney, Australia)

COLONY ASSAYS

After 48 h IFI 16-transfected MCF-7 cells were selected using geneticin (1 mg/ml) (Invitrogen Pty., Mt Waverley, Australia) Selection was maintained for 14 days after which colonies (>50 cells) were fixed, stained with crystal violet and counted.

CELL PROLIFERATION ASSAYS

Cultured cells were brought into suspension using trypsin and counted using a Sysmex CDA 500 system.

CELL CYCLE ANALYSIS

2×10^5 cells were washed (×2 PBS), fixed/permeablised in PBS/70% ethanol and washed (2× PBS), resuspended in citrate buffer (0.1% sodium citrate 0.1% Triton X-100, 50 μg/ml propidium iodide and 100 μg/ml RNaseA) and incubated for 30 min at 37°C. Cells were analysed by Flow cytometry (FACScalibur, BD Biosciences, Franklin Lakes, NJ,) and FCS Express2.0 software (De Novo Software, Ontario, Canada).

WESTERN BLOTTING

Cells were harvested, washed (2× PBS) then resuspended in Lysis Buffer (25 mM Tris pH 7.5, 125 mM NaCl, 2.5 mM EDTA, 0.05% SDS, 0.5% NP40, 0.5% Sodium deoxycholate, 10% glycerol) for 15 min, sonicated (4°C 2× 15 s pulses) and cleared by centrifugation (13,000 rpm, 4°C for 10 min). Western blotting was performed as previously described [Clarke et al., 2003].

IN VITRO KINASE ASSAYS

Cells were washed, (2× PBS), resuspended in lysis buffer (25 mM Tris pH 7.5, 125 mM NaCl, 2.5 mM EDTA, 0.05% SDS, 0.5% NP40, 0.5% sodium deoxycholate, 10% glycerol) for 15 min, sonicated (2× 15 s pulses at 4°C) and cleared by centrifugation (20,000*g*, 4°C for 10 min). Lysates were pre-cleared using a control rabbit polyclonal antibody and Cdk2 was immuno-precipitated using specific antibody and protein A linked to sepharose (Amersham Biosciences, Castle Hills, NSW, Australia). Immunoprecipitates were washed (5× lysis buffer; 2× kinase assay buffer (20 mM Tris pH 7.5, 1.5 mM $MgCl_2$, 1 mM DTT)) and resuspended in kinase assay buffer containing 20 μM ATP, 5 μg Histone H1 and 5 μCi γ-^{32}P-labelled ATP/reaction. After 30 min at 37°C the reaction was stopped by addition of SDS sample buffer and boiling. Proteins were separated by SDS–PAGE and phosphorylated substrates detected by autoradiography.

SENESCENCE ASSOCIATED β-GALACTOSIDASE ASSAYS

Assays were performed as previously described [Dimri et al., 1995]. Numbers of blue cells/field were determined as a proportion of the total. A minimum of 5 fields were counted equating to ~300 cells/treatment.

TELOMERASE ACTIVITY ASSAYS

Lysates were prepared in a 200 μl volume (10 mM Tris pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.5% CHAPS 10% Glycerol at 4°C for 30 min), cleared by centrifugation (20,000*g*, 4°C for 20 min). Two microgram of extracts and serial dilutions were added to telomerase reaction buffer (20 mM Tris pH 8.3, 1.5 mM $MgCl_2$, 68 mM KCl, 0.05% Tween 20, 1 mM EGTA, 100 μM dNTPs), M2 oligo (5′-AATCCGTCGAGCAGAGTT-3′), CX oligo (5′-CCCTTACCCTTACCCTTACCCTAA-3′) (GeneWorks Pty. Ltd, Hindmarsh, Australia), Taq 2U diluted to 50 μl H_2O) and incubated (RT 30 min). Products were amplified by PCR (1 cycle at 94°C for 2 min; 30 cycles at 94°C for 10 s, 50°C for 25 s, 72°C for 30 s; 1 cycle at 94°C for 15 s, 50°C for 25 s, 72°C for 1 min), separated by non-denaturing polyacrylamide, stained with ethidium bromide and analysed using a Bio-rad FX laser scanner and Quantity one software (Bio-Rad, Hercules, CA,).

IN VITRO BINDING ASSAYS

GST fusion proteins were as previously described [Johnstone et al., 2000]. ^{35}S-labelled hTERT was prepared from pCI hTERT by in vitro transcription and translation (IVTT) using a reticulocyte lysate kit (Promega, Madison, WI) and ^{35}S-labeled methionine (ICN Biomedicals, Seven Hills, NSW, Australia) according to the manufacturers instructions. GST proteins bound to glutathione-agarose beads (Sigma, St. Louis, MO) were washed (2× NET-50 (20 mM Tris pH 8.0, 1 mM EDTA, 50 mM NaCl)) for 15 min at room temperature and were

incubated with ^{35}S-labelled IVTT hTERT (2 h at 4°C). Immobilised proteins were washed (5× NET-50), boiled in SDS sample buffer, separated by SDS–PAGE and detected by autoradiography.

PREPARATION OF GENOMIC DNA AND TELOMERE LENGTH ANALYSIS

2×10^5 cells were resuspended in 0.8 ml lysis buffer (50 mM Tris–HCl pH 8, 100 mM EDTA 100 mM NaCl 1% SDS) for 16 h at 56°C. 0.28 ml saturated NaCl was added (5 min at 4°C) and lysates cleared by centrifugation (20,000*g*, 4°C for 10 min). gDNA was precipitated by mixing 0.8 ml of supernatant with 0.6 ml isopropanol, collected by centrifugation (20,000*g*, 4°C for 10 min), washed with 70% ethanol and dried and resuspended (20 mM Tris–HCl pH 8, 1 mM EDTA). gDNA (3 μg) was digested with *Rsa*I and *Hin*fI, separated by agarose electrophoresis and transferred to Hybond N+ membranes as previously described [Sambrook et al., 1989]. Membranes were pre-hybridised (6 h at 42°C), hybridised with ^{32}P-labelled TEL1 oligo 5′-TTAGGGTTAGGGTTAGGG-3′ (16 h at 42°C) then washed (Twice 2× SSC 25°C, Twice 2× SSC 0.1% SDS 25°C, Once 2× SSC 0.1% SDS 42°C) before exposure to X-Ray film (Kodak).

IMMUNOFLUORESCENCE AND MICROSCOPY

Cells were fixed and permeablised using 4% paraformaldehyde/0.1% TX100 dissolved in PBS. Blocked with 1% BSA and stained with primary antibody and counterstained with Alexa594 or Alexa488 conjugated anti-mouse immunoglobulin. Images were captured using Zeiss Axioscope or a Biorad MRC-1024 laser scanning confocal microscope. Image analysis was performed using SpotAdvanced, ImageJ and Adobe Photoshop software.

CO-IMMUNOPRECIPITATIONS

Cells were washed, (×2 PBS), resuspended in Lysis Buffer (25 mM Tris pH 8, 150 mM NaCl, 1% NP40) for 15 min and cleared by centrifugation (20,000*g*, 4°C for 10 min). IFI 16 was immunoprecipitated using specific rabbit polyclonal antibody and protein A linked to sepharose (Amersham Biosciences, Castle Hills, NSW, Australia); rabbit polyclonal antibodies were used as a control. Immunoprecipitates were washed (5× lysis buffer) then boiled in SDS sample buffer.

RESULTS

ALL THE SPLICE ISOFORMS OF IFI 16 INHIBIT COLONY FORMATION

The three isoforms of IFI 16 that are produced by alternative splicing within the S/T/P rich sequences between the 200 A and B repeats are present in all IFI 16-expressing cell lines tested [Johnstone et al., 1998]. We tested whether these isoforms could inhibit cell proliferation by transfecting either the A B or C isoforms of IFI 16 or an empty vector control into MCF-7 cells and performing colony assays. MCF-7 were chosen because they do not express endogenous IFI 16, but express *wild type* alleles of pRb and p53 that are reported to be important for the growth suppressive activity of HIN-200 proteins [Hertel et al., 2000; Johnstone et al., 2000; Fujiuchi et al., 2004]. Each isoform was expressed at the expected size (Fig. 1A) and localised to the nucleus (Fig. 1B). Following stable transfection and 2 weeks of antibiotic selection, over 1,000 colonies were recovered from cultures transfected with control plasmid, but 80–90% fewer colonies were apparent in cultures transfected with any of the IFI 16 isoforms (Fig. 1C).

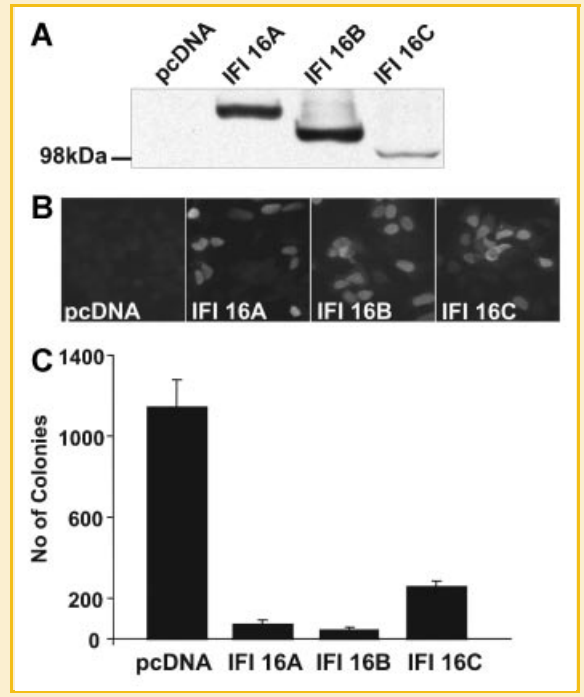

Fig. 1. Expression of IFI 16 inhibits colony formation in MCF-7 cells. 2×10^5 MCF-7 cells were plated on 10 cm petri dishes and transfected with pcDNA3 encoding the A, B or C isoforms of IFI 16 (IFI 16 A, B or C) or pcDNA3 empty vector (pcDNA) as a control. Expression and sub-cellular localisation of the IFI 16 isoforms was assessed 48 h after transfection using 1G7 mAb by (A) Western blot or (B) immunofluorescence staining and microscopy. C: After 48 h cells were selected with 1 mg/ml G418 and after a further 2 weeks colonies were stained with crystal violet and counted. Histograms show mean and standard deviation of triplicates from a single experiment representing three independent experiments.

INDUCIBLE NUCLEAR EXPRESSION OF AN ER-IFI 16 FUSION PROTEIN SUPPRESSES CELL PROLIFERATION AND TRIGGERS G1 PHASE CELL CYCLE ARREST

We were unable to recover any IFI 16-expressing clones after stable transfection. Therefore we chose to express the IFI 16 B isoform, as an estrogen receptor ligand binding domain (ER) fusion protein. In the absence of ligands ER fusion proteins are frequently sequestered in the cytoplasm [Coghill et al., 2001]. ER-IFI 16 localised to the cytoplasm of MCF-7 cells (Fig. 2A), but treatment of cells for 2 h with 4OHT caused it to translocate to the nucleus similar to the localisation of wild type IFI 16 (see Fig. 1B). Cytoplasmic to nuclear translocation of ER-IFI 16 occurred in a dose-dependent manner following 4OHT treatment (Fig. 2A). Translocation of ER-IFI 16 to the nucleus began less than 2 min after treatment of cells with 100 nM of 4OHT, and was complete by 30 min (Fig. S2A). Western blotting confirmed ER-IFI 16 was expressed at the expected size and was efficiently translocated from cytosol to nucleus (Fig. S2B).

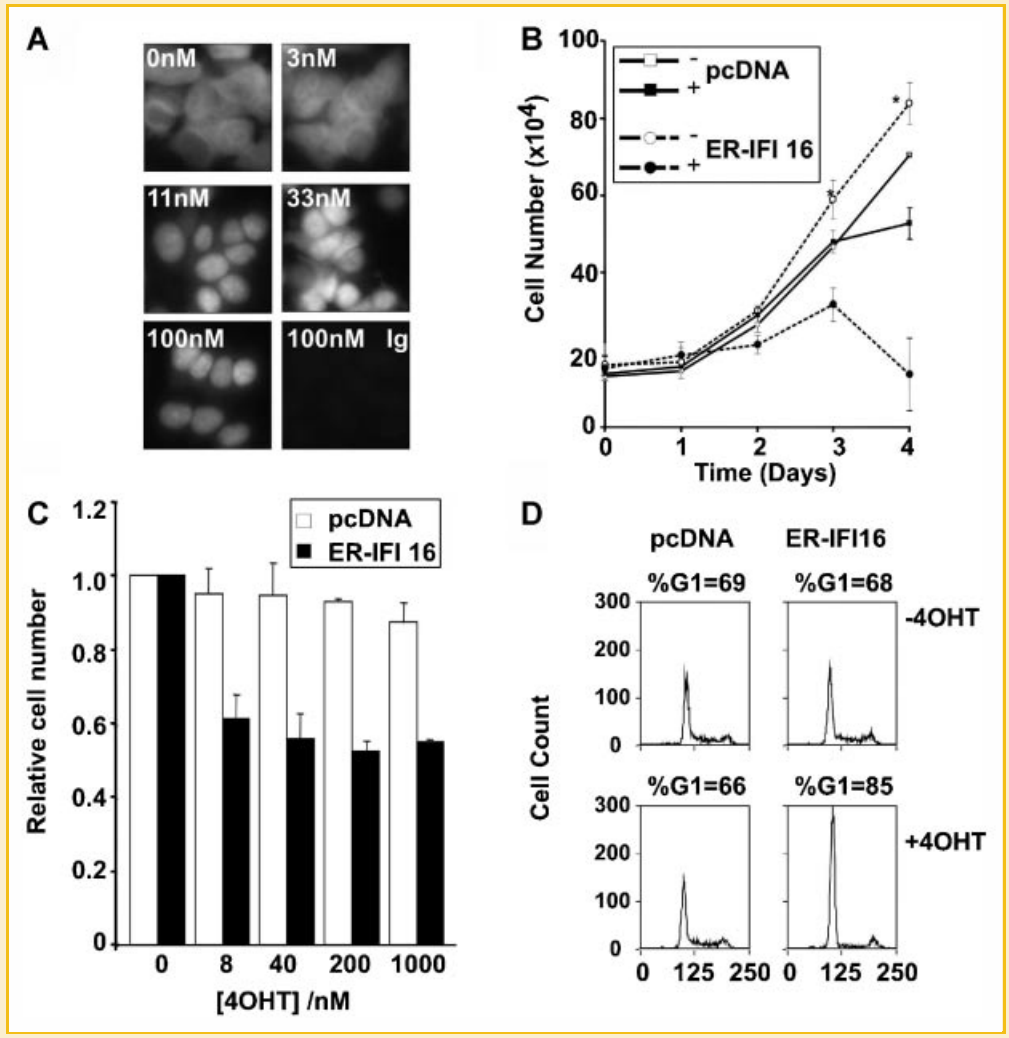

Fig. 2. Expression of ER- IFI 16 in the nucleus induces a decrease in cell proliferation. A: ER-IFI 16 expressing MCF-7 cells were treated for 120 min with indicated concentrations of 4OHT. Cells were stained with mAb 1G7, or a control isotype matched mAb (Ig) and sub-cellular localisation was analysed by immunofluorescence microscopy. B: 2×10^4 MCF-7 cells expressing ER-IFI 16 (dotted lines) or pcDNA control plasmid (solid lines) were treated in the presence (+) or absence (−) of 100 nM 4OHT for different times. Cells were harvested and counted after each day of treatment. Results show the mean and standard error of five independent experiments ($^*P < 0.02$ between control and 4OHT treated ER-IFI 16 expressing cells; Student's paired *t*-test). C: 2×10^4 control (open bars) or ER-IFI 16 expressing (closed bars) MCF-7 cells were treated for 72 h with different doses of 4OHT. Cells were harvested and counted. Results represent the mean and standard error of triplicate cultures and are representative of 3 independent experiments. D: 2×10^5 ER-IFI 16 expressing MCF-7 cells were treated with 100 nM 4OHT. Cells were harvested, washed with PBS, fixed and permeablised using 70% ethanol/30% PBS after each day of treatment. DNA content was assessed by staining with propidium iodide and flow cytometry. The percentage of cells in G1 phase of the cell cycle is indicated.

When the ER-IFI 16 expressing cells were cultured in the presence of 4OHT a proliferative defect was apparent (Fig. 2B). Small differences in cell number were already observed after 24 h treatment with 4OHT, but these became more marked between 72 and 96 h ($P < 0.02$). Similar effects were observed in two other independently derived ER-IFI 16 expressing clones (Fig. S3A,B). The majority of the anti-proliferative activity was triggered by doses as low as 8 nM (Fig. 2C), correlating well with the doses of 4OHT that induced nuclear localisation of ER-IFI 16 (Fig. 2A).

The reduction in cell number following nuclear translocation of ER-IFI 16 may have been caused by induction of apoptosis, or retardation of the cell cycle. We studied this possibility by fixing 4OHT treated cells, staining them with propidium iodide then analysing DNA content by flow cytometry. In ER-IFI 16 expressing cells a reduction in S phase and a striking increase in cells with 2N DNA were observed following 72 h 4OHT treatment (Fig. 2D). These are indicators of cell cycle arrest in the G1 phase. We did not observe any consistent alteration in cells with <2 N DNA content in any cell lines or treatment groups. Moreover, we did not detect expression of phosphatidylserine on the outer cell membrane of ER-IFI 16-expressing cells treated with 4OHT as assessed by annexin V staining (data not shown). Taken together, this indicated that cell cycle arrest rather than induction of apoptosis explained the reduction in cell number we observed following nuclear localisation of IFI 16.

ER-IFI 16-INDUCED CELL CYCLE ARREST DOES NOT INVOLVE ALTERED EXPRESSION OR ACTIVITY OF P53, P21$^{WAF1/CIP1}$, CDK2 OR PRB

Our initial studies of molecular mechanisms of IFI 16-induced cell cycle arrest focused on proteins that have previously been associated with HIN-200 proteins and arrest in the G1 phase of the cell cycle [Lembo et al., 1995, 1998]. Cells induced to express nuclear ER-IFI 16 showed no change in the levels of p53 or p21$^{WAF1/CIP1}$ (Fig. 3A). However, treatment of MCF-7 cells with γ-irradiation caused a marked induction of both p53 and p21$^{WAF1/CIP1}$ indicating that the p53/p21$^{WAF1/CIP1}$ pathway is intact in the cells used in our study (Fig. 3A). Treatment of ER-IFI 16-expressing cells with 4OHT did not alter the phosphorylation status of pRb or the kinase activity of Cdk2 (Fig. 3B,C). In contrast, when G1 cell cycle arrest was triggered by serum starvation in these cells, Cdk2 kinase activity was reduced and hypophosphorylation of pRb was observed (Fig. 3B,C). We concluded that in our system, IFI 16-induced cell cycle arrest in G1 does not occur due to p53-mediated activation of *CDKN1A* (encoding p21$^{WAF1/CIP1}$) or hypophosphorylation of pRb.

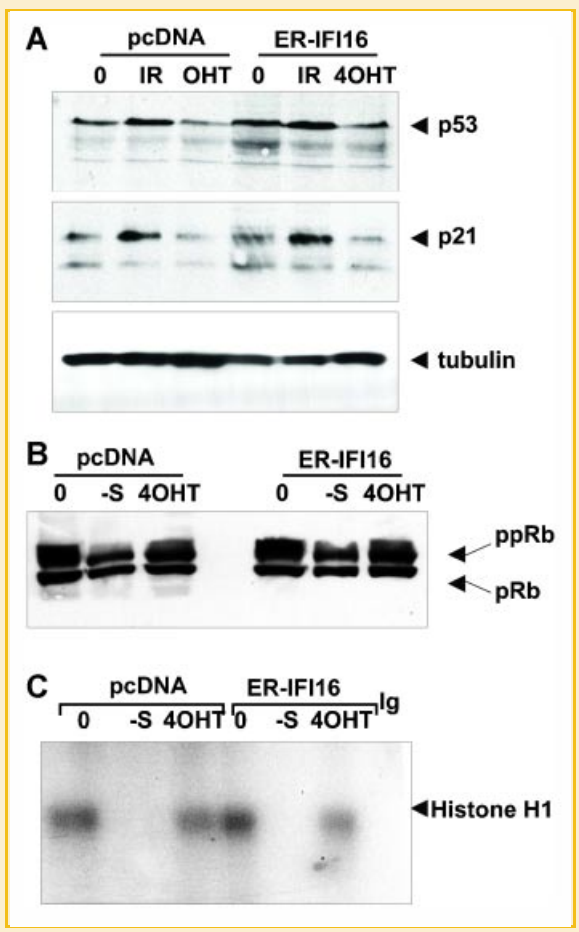

Fig. 3. Translocation of IFI 16 to the nucleus does not affect expression of p53, or p21 and phosphorylation of pRb and activity CDK2. A: 2×10^5 MCF-7 cells expressing ER-IFI 16 or pcDNA were treated ±100 nM 4-OHT (4OHT) for 72 h or 5Gy γ-irradiation (IR) for 4 h. p53, p21 and tubulin in lysates were analysed by SDS-PAGE and Western blotting. B: 2×10^5 MCF-7 cells expressing ER-IFI 16 or pcDNA were treated ±100 nM 4-OHT (4OHT) or deprived of serum (−S) for 72 h. pRb in lysates was analysed by SDS-PAGE and Western blotting. C: 2×10^5 MCF-7 cells expressing ER-IFI 16 or pcDNA were treated ±100 nM 4-OHT (4OHT) or deprived of serum (−S) for 72 h. Cells were lysed and CDK2 was purified from these extracts by immunoprecipitation and in vitro kinase assays performed using histone H1 as a substrate. Control immunoprecipitations and kinase assays were performed using lysates prepared from untreated control cells using a non-specific antibody (Ig).

IFI 16-INDUCED CELL CYCLE ARREST COINCIDES WITH THE APPEARANCE OF A SENESCENCE-LIKE PHENOTYPE AND INHIBITION OF TELOMERASE

The expression of IFI 16 correlated with a senescent phenotype in cultures of normal fibroblasts [Xin et al., 2003, 2004]. We used our inducible system to determine whether IFI 16 expression was responsible for such phenotypes. After 7 days of treatment with 4OHT, ER-IFI 16-expressing cells assumed a distinctive, flattened morphology compared to untreated cells (Fig. 4A), but no alteration in the morphology was observed in control cells following treatment with 4OHT. Senescence-associated β-galactosidase activity was detected in a substantial proportion of these cells expressing nuclear IFI 16 (Fig. 4A). These data suggest that nuclear expression of ER-IFI 16 is sufficient to induce a senescence-like phenotype. We reasoned that IFI 16 may trigger biochemical events that overlapped with those related to replicative senescence and therefore we determined the effect of IFI 16 on telomerase activity. Telomere amplification repeat (TRAP) assays were performed using serial dilutions of extracts from cells. Differences in activity between samples were identified by determining the dilutions at which TRAP activity could be titrated away. At a 1:36 dilution of extracts, telomerase activity was similar in control cells treated in the presence and absence of 4OHT. However, in ER-IFI 16-expressing cells at a 1:36 dilution of extracts 4OHT treatment caused a 50% reduction in telomerase activity (Figs. 4B,C and S4). Serum-starvation did not affect telomerase activity (Fig. 4B) although it did induce cell cycle arrest in the G1 phase (data not shown). To determine whether telomere shortening was occurring, genomic DNA was isolated from control and ER-IFI 16-expressing cells treated in the presence and absence of 4OHT, fragmented using restriction enzymes and analysed by Southern blot using radiolabelled probes complementary to telomeres. We did not observe any gross changes in telomere length when either control or ER-IFI 16 expressing cells were treated with 4OHT (Fig. 4D) suggesting that the senescence-like state was not triggered by telomere shortening.

REGIONS OF IFI 16 NECESSARY FOR REGULATION OF TELOMERASE ACTIVITY

We performed structure function analysis of IFI 16 using a series of point and truncation mutants of IFI 16 (Table I). The ER-IFI 16 (1–477) protein lacking the 200 B domain of IFI 16 localised to the nucleus following 4OHT treatment and suppressed cell proliferation and telomerase activity suggesting the 200 B domain was dispensable for the activity of IFI 16. Similarly point mutants S128A and S143A, that were predicted to affect nuclear localisation of IFI 16 [Briggs et al., 2001] localised to the nucleus following 4OHT treatment and inhibited cell proliferation and telomerase activity. However, a T235I/T237I mutant of IFI 16 that did

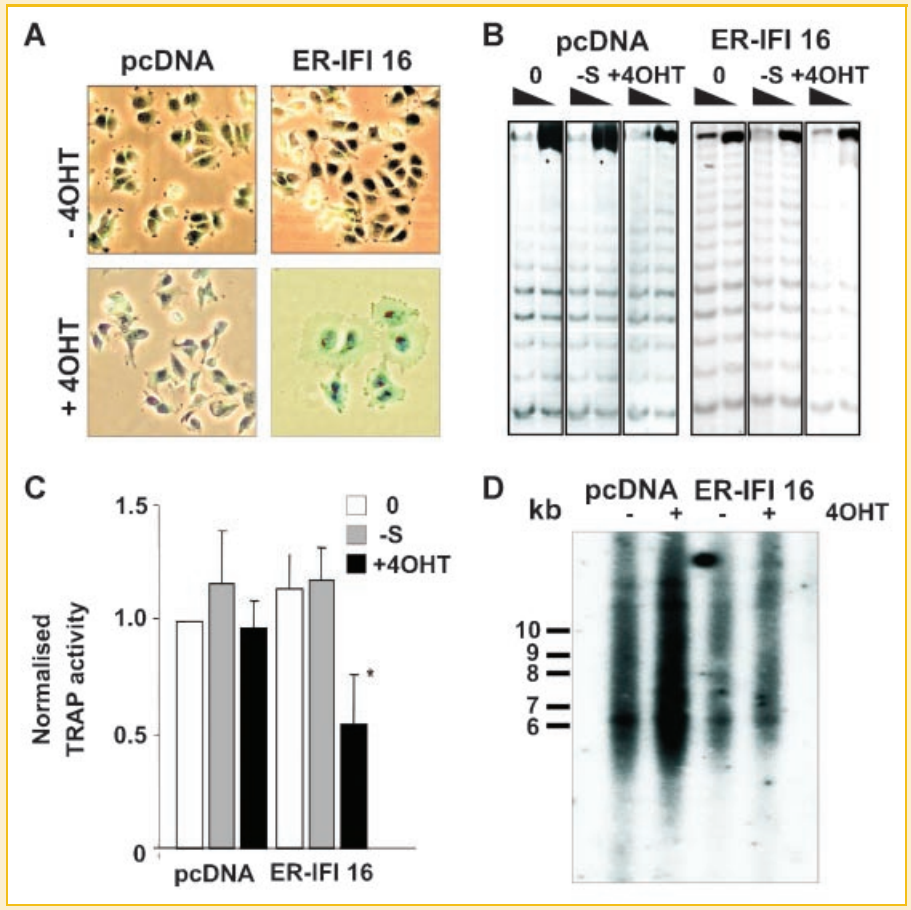

Fig. 4. Translocation of IFI 16 to the nucleus leads to a senescence-like morphology, induces senescence associated β-galactosidase and inhibits TRAP activity without affecting telomere length. A: 2×10^4 ER-IFI 16 expressing MCF-7 cells were treated ±100 nM 4OHT for 7 days, stained with X-gal for senescence-associated β-galactosidase activity and analysed by phase contrast microscopy. B: 2×10^5 ER-IFI 16 expressing MCF-7 cells were treated ±100 nM 4OHT (+4OHT) or deprived of serum (−S) for 72 h, cell lysates were assayed for telomerase activity using the TRAP assay. Activity was assayed in neat extracts and in sixfold serial dilutions with decreasing extract concentration denoted by filled triangles. C: TRAP activity was quantified using QuantityOne software, normalised to untreated controls and statistics analysed using Origin7 software. Histograms show the mean and standard deviation of three independent experiments. ($^*P < 0.05$ between control and 4OHT treated ER-IFI 16 expressing cells; Student's paired *t*-test). D: 2×10^5 MCF-7 cells expressing ER-IFI 16 or pcDNA were treated ±100 nM 4OHT for 72 h. Genomic DNA was prepared from these cells, digested with *Rsa*I and *Hin*fI and analysed by Southern blotting using radio-labelled probes complementary to telomeric repeats. Results were visualised by autoradiography.

not translocate to the nucleus following 4OHT treatment did not affect proliferation of cells or TRAP activity. These data support a direct link between localisation of IFI 16 to the nucleus, its ability to inhibit TRAP activity and cellular proliferation.

hTERT AND IFI 16 CO-LOCALISE AND PHYSICALLY INTERACT

Endogenous hTERT is expressed at very low levels and therefore we assessed the possible interaction between IFI 16 and hTERT using overexpressed proteins. To determine whether IFI 16 and hTERT co-localised, we transfected IFI 16 into hTERT-overexpressing MCF-7

TABLE I. Comparison of Various Truncation and Point Mutants of IFI 16 on Assays for Sub-Cellular Localisation, Cellular Proliferation and Telomerase Activity

	Localisation		Proliferation		TRAP activity	
Mutant	−4OHT	+4OHT	−4OHT	+4OHT	−4OHT	+4OHT
aa1-729	Cyto	Nuc	+++	−	+++	+
aa1-477	Cyto	Nuc	+++	−	+++	−
aa1-729 (S128A)	Cyto	Nuc	+++	−	+++	+
aa1-729 (S128A/S143A)	Cyto	Nuc	+++	−	+++	+
aa1-729 (T235I/T237I)	Cyto	Cyto	+++	+++	+++	+++

ER-fusion proteins with full length IFI 16 (aa1–729), a mutant lacking the 200 B domain (aa1–477) and various point mutants were each assessed for (i) their sub-cellular localisation by immunofluorescence and microscopy (cyto, cytoplasmic; nuc, nuclear), (ii) their ability to inhibit cellular proliferation and (iii) their ability to inhibit telomerase activity by TRAP assay.

cells and studied the localisation of each protein by immunofluorescence and confocal microscopy. In cells expressing IFI 16, both proteins were expressed diffusely in the nucleus (Fig. 5A,B), and when expression of the two proteins was detected in the same cells, there was a substantial degree of co-localisation between IFI 16 and hTERT (Fig. 5C) suggesting these two proteins might interact. Control staining confirmed the specificity of each antibody (Fig. 5D–L). We assessed the ability of IFI 16 and hTERT to interact in vitro by mixing ^{35}S-labelled, in vitro transcribed/translated (IVTT) hTERT with GST-IFI 16 or GST immobilised on glutathione-coated sepharose beads. ^{35}S-labelled hTERT could only be detected bound to immobilised GST-IFI 16, not GST (Fig. 6A). The interaction between IFI 16 and hTERT was also demonstrated by co-immunoprecipitation from intact cells. IFI 16 and hTERT were detected in lysates of cells co-transfected with these proteins (Fig. 6B) and subsequently IFI 16 was immunprecipitated from these lysates using a rabbit polyclonal antiserum and the immobilised proteins were analysed by Western blot. The presence of IFI 16 was confirmed by an initial Western blot and subsequently, the membrane was probed using antibodies against hTERT. Bands co-migrating with those of IFI 16 and hTERT were detected in these immunoprecipitates (Fig. 6B) suggesting that IFI 16 and hTERT physically interact. When the site of interaction was mapped using a series of IFI 16 truncation mutants (Fig. 6C) IVTT hTERT only bound to fragments corresponding to the N-terminus of IFI 16 (1–159) (Fig. 6D) suggesting the physical interaction between IFI 16 and hTERT requires the N-terminal DAPIN/PAAD/Pyrin domain of IFI 16. This is consistent with our findings that truncations at the C-terminus of IFI 16 did not affect its ability to suppress cell proliferation or telomerase activity.

DISCUSSION

We used a novel, inducible, system of regulating the sub-cellular localisation of IFI 16 to investigate the growth-suppressive activity of the protein. This system was of great utility as it provided an

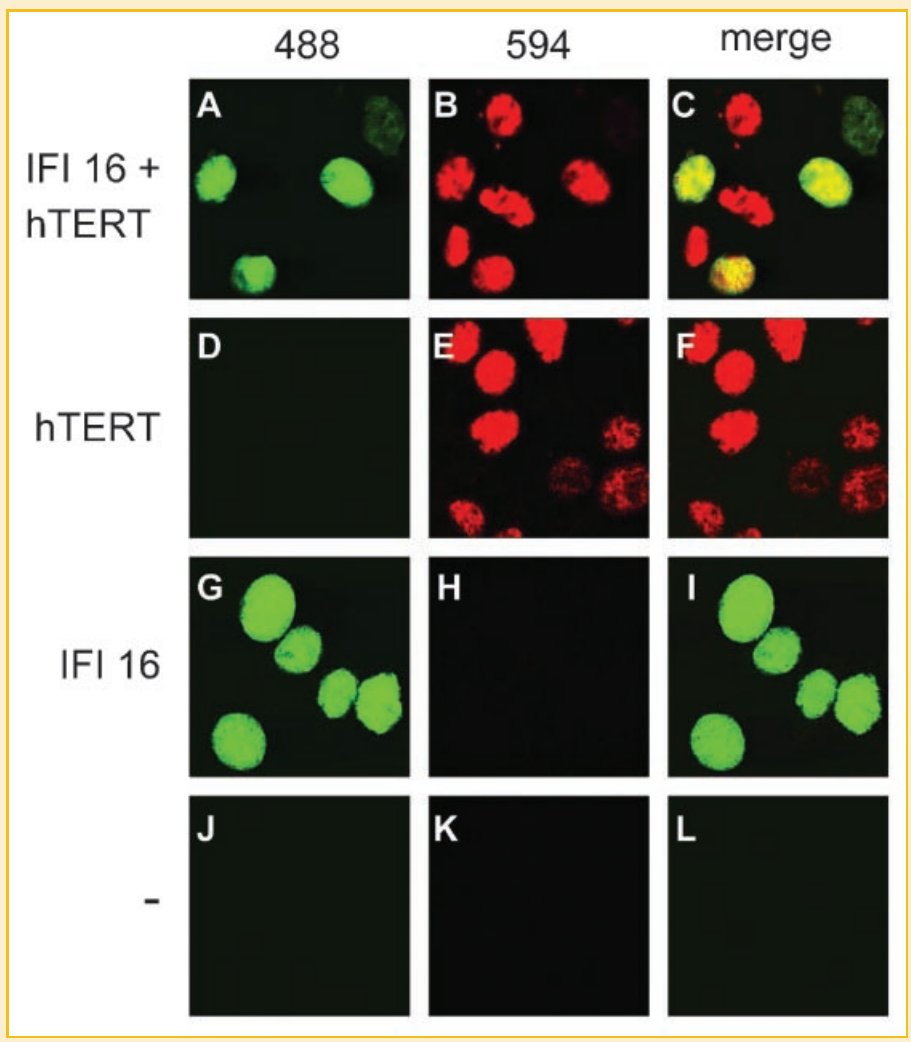

Fig. 5. IFI 16 and hTERT co-localise in the nucleus. MCF-7 cells stably over-expressing hTERT and transiently transfected with IFI 16 were fixed and unstained (–), stained with mouse mAb IFI 16 (IFI 16), goat polyclonal Ab (hTERT) or both (hTERT + IFI 16). All samples were counterstained with anti-mouse Alexa 488 and anti-goat Alexa 594. Images displayed were captured in green (A,D,G,J) or red (B,E,H,K) channels and merged (C,F,I,L).

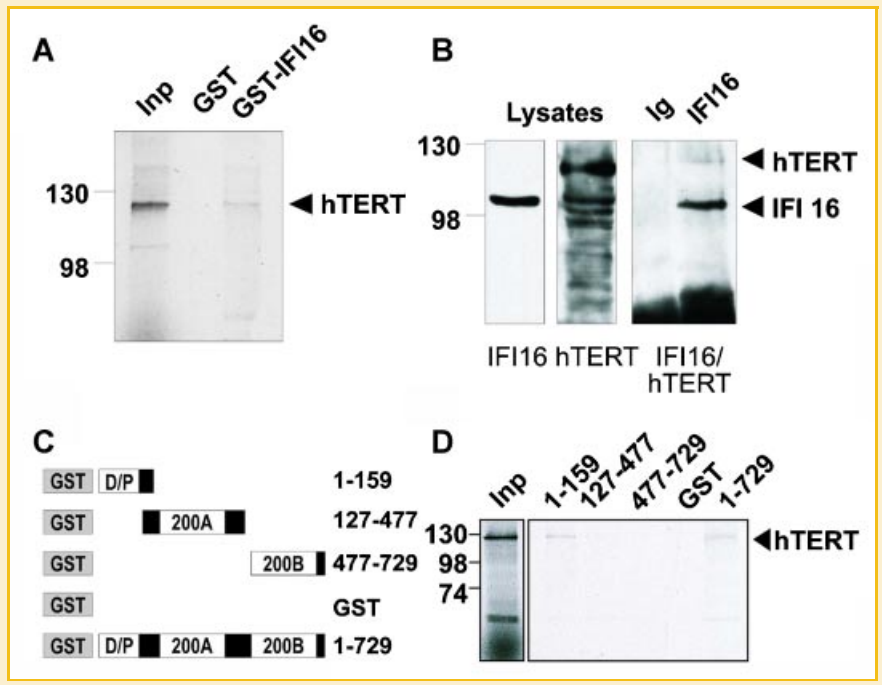

Fig. 6. IFI 16 and hTERT interact through the PAAD/DAPIN/Pyrin domain of IFI 16. A: ^{35}S-labelled IVTT hTERT was immobilised on glutathione-sepaharose beads linked to GST or GST-IFI 16. Interacting proteins were separated by SDS–PAGE and hTERT binding was assessed by autoradiography relative to input (Inp) (50% of binding reaction). B: 293 cells were co-transfected with pcDNA IFI 16B and pCI hTERT and harvested after 48 h and expression of IFI 16 and hTERT was confirmed in lysates. IFI 16 was immunoprecipitated (IP) using a specific rabbit pAb (IFI 16) or control antisera (Ig) from whole cell lysates. Purified IFI 16 was detected initially by Western blot (IB), the blot was not stripped and then antibodies to hTERT were added and detected by enhanced chemiluminescence. C: Schematic diagram of IFI 16 GST fusion protein and truncation mutants showing the DAPIN/PAAD (D/P) domains and the 200× domains (200 A and 200 B). D: ^{35}S-labelled IVTT hTERT was incubated with GST, GST-IFI 16 or GST fusion proteins of IFI 16 truncation mutants immobilised on glutathione-sepharose beads. Interacting proteins were separated by SDS–PAGE and bound hTERT was assessed by autoradiography relative to an input control (Inp) (50% of binding reaction).

opportunity to stably express IFI 16 and 'activate' it acutely by regulating its localisation. We confirmed previous observations that IFI 16 and related proteins trigger cell cycle arrest in G1 [Lembo et al., 1995, 1998; Xin et al., 2004]. This phenotype was specific to IFI 16 as it was not observed when a point mutant of IFI 16 was tested. We did not detect any effect of IFI 16 upon the p53/p21$^{WAF1/CIP1}$/Cdk2/pRb cell cycle regulatory axis, but this may because of differences between our experimental system and those used previously [Kwak et al., 2003; Fujiuchi et al., 2004]. Our studies revealed that expression of IFI 16 was sufficient to induce a senescence-like phenotype. Moreover, IFI 16 co-localised with hTERT in vivo, bound to hTERT in vivo and in vitro and telomerase activity was markedly reduced in cells induced to express nuclear IFI 16. We demonstrated an association between the nuclear expression of IFI 16, abrogation of telomerase activity and suppression of cell proliferation. These studies identify a potentially novel mechanism of action of IFI 16 in regulating cell proliferation through a functional interaction with hTERT. A "dominant negative" mutant of p204, the murine HIN-200 family member most closely related to IFI 16, has been reported to increase TRAP activity [De Andrea et al., 2002] suggesting that the phenomena we describe may be broadly applicable to the HIN-200 family of proteins.

The growth suppression, morphological changes, expression of SA-β-galactosidase activity and reduced telomerase activity that we observed are all characteristic of replicative senescence [Dimri et al., 1995]. The induction of senescence we observed following acute activation of ER-IFI 16 would explain why we were unable to propagate cells overexpressing wild type IFI 16. IFI 16 has previously been linked to replicative senescence by expression analysis that indicated increased levels of IFI 16 were associated with a senescence-like phenotype and that the onset of senescence was affected by transfection with siRNA targeting IFI 16 [Xin et al., 2003, 2004]. Replicative senescence is triggered by critical shortening of telomeres [Bodnar et al., 1998] after progressive telomere attrition over periods of weeks to months, depending on initial telomere length [Harley et al., 1990]. However, we observed that retarded cell proliferation occurred between 24 and 48 h after nuclear translocation of ER-IFI 16 which limits the opportunity for telomere erosion. Moreover, we found no evidence that average telomere length had altered, but can not exclude the possibility that individual telomeres may have become critically shortened leading to the phenomenon we observed. It is unlikely that "oncogene-induced senescence" occurs, as we did not observe the elevated levels of p53 and hypo-phosphorylation of pRb characteristic of this process [Braig et al., 2005]. Some pharmacological agents that target telomerase modulate cell proliferation within 24–48 h [El-Daly et al., 2005] and loss of proliferation of cell lines transfected with hTR siRNA is evident within 1 week [Li et al., 2005]. Like ER-IFI 16 expressing cells, the phenotype of cells transfected with hTR siRNA did not resemble replicative or oncogene-induced senescence

because loss of cellular proliferation was not linked to changes in telomere length, did not depend on p53 and did not involve a DNA damage response. The molecular events that underlie rapid loss of proliferation caused by endogenous (e.g. ER-IFI 16) and exogenous (e.g. siRNA targeting hTR/hTERT and chemical) telomerase inhibitors remain largely uncharacterised. Microarray technology was used to identify the ''transcriptional signature'' of hTR siRNA-knockdown [Li et al., 2005]. However, it is not known if, or how, the genes identified contribute to reduced telomerase activity and reduced proliferation. It will be of interest to determine whether ER-IFI 16 elicits a similar transcriptional response to that of hTR siRNA.

Our data supports the existence of telomere length-independent functions for telomerase. Other evidence in favour of such functions includes observations that overexpression of TERT increased proliferation of cells in vitro [Stewart et al., 2002] and the incidence of mammary tumours [Artandi et al., 2002], under circumstances where telomere length is not limiting. In addition, hTERT can act in the absence of hTR because expression of hTERT caused hyper-proliferation of keratinocytes in the bulb of the hair follicle in hTR−/− animals [Sarin et al., 2005]. These studies suggest TERT plays a role in cellular proliferation that is independent of its telomere length maintenance activity and IFI 16 is the first endogenous factor reported to affect this activity.

Efforts to identify the true physiological role of HIN-200 proteins are yet to reach any definitive conclusions [Ludlow et al., 2005]. Although AIM2 acts as a cytoplasmic DNA sensor involved in the activation of the NALP3 inflammasome through an interaction with ASC [Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009], other HIN-200 proteins are not found the cytoplasm and did not share this activity. Our previous studies have shown IFI 16 to be expressed in tissues with a high proliferative potential such as the haemopoietic and epithelial compartments [Wei et al., 2003]. Interestingly, this expression pattern corresponds with that of hTERT expression/activity [Forsyth et al., 2002]. As hTERT expressing cells have full proliferative capacity, IFI 16 does not fully compromise hTERT activity in these cells possibly because it does not interact with all the telomerase complexes in the cell. Recently, the components of active telomerase complexes were identified [Cohen et al., 2007] and did not include IFI 16 thus we hypothesise that the pool of hTERT bound by IFI 16 is inactive, whilst those molecules not bound remain capable of telomere extension. This model predicts that IFI 16 acts as an endogenous regulator of telomerase activity.

The ability of various HIN-200 proteins to inhibit cellular proliferation and induce senescence and/or apoptosis has led to the proposal they may be exploited for use in gene therapy to treat cancers. This has been demonstrated using the murine family member p202 in a xenograft model [Ding et al., 2002], and subsequently using the human family member IFIX [Ding et al., 2004]. It is possible that IFI 16 could also be used in such an approach, but specific and efficient targeting to the tumour would be required.

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REFERENCES

Artandi SE, Alson S, Tietze MK, Sharpless NE, Ye S, Greenberg RA, Castrillon DH, Horner JW, Weiler SR, Carrasco RD, DePinho RA. 2002. Constitutive telomerase expression promotes mammary carcinomas in aging mice. Proc Natl Acad Sci USA 99:8191–8196.

Asefa B, Klarmann KD, Copeland NG, Gilbert DJ, Jenkins NA, Keller JR. 2004. The interferon-inducible p200 family of proteins: A perspective on their roles in cell cycle regulation and differentiation. Blood Cells Mol Dis 32:155–167.

Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE. 1998. Extension of life-span by introduction of telomerase into normal human cells. Science 279:349–352.

Braig M, Lee S, Loddenkemper C, Rudolph C, Peters AH, Schlegelberger B, Stein H, Dorken B, Jenuwein T, Schmitt CA. 2005. Oncogene-induced senescence as an initial barrier in lymphoma development. Nature 436:660–665.

Briggs LJ, Johnstone RW, Elliot RM, Xiao CY, Dawson M, Trapani JA, Jans DA. 2001. Novel properties of the protein kinase CK2-site-regulated nuclear- localization sequence of the interferon-induced nuclear factor IFI 16. Biochem J. 353 (Pt 1): 69–77.

Clarke CJ, Apostolidis V, Hii LL, Gough DJ, Trapani JA, Johnstone RW. 2003. Critical role of the transcription factor AP-1 for the constitutive and interferon-induced expression of IFI 16. J Cell Biochem 89:80–93.

Coghill E, Eccleston S, Fox V, Cerruti L, Brown C, Cunningham J, Jane S, Perkins A. 2001. Erythroid Kruppel-like factor (EKLF) coordinates erythroid cell proliferation and hemoglobinization in cell lines derived from EKLF null mice. Blood 97:1861–1868.

Cohen SB, Graham ME, Lovrecz GO, Bache N, Robinson PJ, Reddel RR. 2007. Protein composition of catalytically active human telomerase from immortal cells. Science 315:1850–1853.

Damm K, Hemmann U, Garin-Chesa P, Hauel N, Kauffmann I, Priepke H, Niestroj C, Daiber C, Enenkel B, Guilliard B, Lauritsch I, Muller E, Pascolo E, Sauter G, Pantic M, Martens UM, Wenz C, Lingner J, Kraut N, Rettig WJ, Schnapp A. 2001. A highly selective telomerase inhibitor limiting human cancer cell proliferation. EMBO J 20:6958–6968.

Dawson MJ, Trapani JA. 1995. IFI 16 gene encodes a nuclear protein whose expression is induced by interferons in human myeloid leukaemia cell lines. J Cell Biochem 57:39–51.

De Andrea M, Ravotto M, Noris E, Ying GG, Gioia D, Azzimonti B, Gariglio M, Landolfo S. 2002. The interferon-inducible gene, Ifi204, acquires malignant transformation capability upon mutation at the Rb-binding sites. FEBS Lett 515:51–57.

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O., et al. 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci USA 92:9363–9367.

Ding Y, Wen Y, Spohn B, Wang L, Xia W, Kwong KY, Shao R, Li Z, Hortobagyi GN, Hung MC, Yan DH. 2002. Proapoptotic and antitumor activities of adenovirus-mediated p202 gene transfer. Clin Cancer Res 8:3290–3297.

Ding Y, Wang L, Su LK, Frey JA, Shao R, Hunt KK, Yan DH. 2004. Antitumor activity of IFIX, a novel interferon-inducible HIN-200 gene, in breast cancer. Oncogene 23:4556–4566.

El-Daly H, Kull M, Zimmermann S, Pantic M, Waller CF, Martens UM. 2005. Selective cytotoxicity and telomere damage in leukemia cells using the telomerase inhibitor BIBR1532. Blood 105:1742–1749.

Fernandes-Alnemri T, Yu JW, Datta P, Wu J, Alnemri ES. 2009. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. Nature 458:509–513.

Folini M, Bandiera R, Millo E, Gandellini P, Sozzi G, Gasparini P, Longoni N, Binda M, Daidone MG, Berg K, Zaffaroni N, Zaffaroni N. 2007. Photochemically enhanced delivery of a cell-penetrating peptide nucleic acid conjugate targeting human telomerase reverse transcriptase: Effects on telomere status and proliferative potential of human prostate cancer cells. Cell Prolif 40:905–920.

Forsyth NR, Wright WE, Shay JW. 2002. Telomerase and differentiation in multicellular organisms: Turn it off, turn it on, and turn it off again. Differentiation 69:188–197.

Fujiuchi N, Aglipay JA, Ohtsuka T, Maehara N, Sahin F, Su GH, Lee SW, Ouchi T. 2004. Requirement of IFI16 for the maximal activation of p53 induced by ionizing radiation. J Biol Chem 279:20339–20344.

Harley CB, Futcher AB, Greider CW. 1990. Telomeres shorten during ageing of human fibroblasts. Nature 345:458–460.

Hertel L, Rolle S, De Andrea M, Azzimonti B, Osello R, Gribaudo G, Gariglio M, Landolfo S. 2000. The retinoblastoma protein is an essential mediator that links the interferon-inducible 204 gene to cell-cycle regulation. Oncogene 19:3598–3608.

Hornung V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, Caffrey DR, Latz E, Fitzgerald KA. 2009. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature 458:514–518.

Johnstone RW, Kerry JA, Trapani JA. 1998. The human interferon-inducible protein, IFI 16, is a repressor of transcription. J Biol Chem 273:17172–17177.

Johnstone RW, Wei W, Greenway A, Trapani JA. 2000. Functional interaction between p53 and the interferon-inducible nucleoprotein IFI 16. Oncogene 19:6033–6042.

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW. 1994. Specific association of human telomerase activity with immortal cells and cancer. Science 266:2011–2015.

Kwak JC, Ongusaha PP, Ouchi T, Lee SW. 2003. IFI16 as a negative regulator in the regulation of p53 and p21(Waf1). J Biol Chem 278:40899–40904.

Lembo D, Angeretti A, Benefazio S, Hertel L, Gariglio M, Novelli F, Landolfo S. 1995. Constitutive expression of the interferon-inducible protein p202 in NIH 3T3 cells affects cell cycle progression. J Biol Regul Homeost Agents 9:42–46.

Lembo M, Sacchi C, Zappador C, Bellomo G, Gaboli M, Pandolfi PP, Gariglio M, Landolfo S. 1998. Inhibition of cell proliferation by the interferon-inducible 204 gene, a member of the Ifi 200 cluster. Oncogene 16:1543–1551.

Li S, Crothers J, Haqq CM, Blackburn EH. 2005. Cellular and gene expression responses involved in the rapid growth inhibition of human cancer cells by RNA interference-mediated depletion of telomerase RNA. J Biol Chem 280:23709–23717.

Littlewood TD, Hancock DC, Danielian PS, Parker MG, Evan GI. 1995. A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nucleic Acids Res 23:1686–1690.

Ludlow LE, Johnstone RW, Clarke CJ. 2005. The HIN-200 family: More than interferon-inducible genes? Exp Cell Res 308:1–17.

Roberts TL, Idris A, Dunn JA, Kelly GM, Burnton CM, Hodgson S, Hardy LL, Garceau V, Sweet MJ, Ross IL, Hume DA, Stacey KJ. 2009. HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. Science 323:1057–1060.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning, A laboratory manual. Cold Spring Harbor, NY: Laboratory Press.

Sarin KY, Cheung P, Gilison D, Lee E, Tennen RI, Wang E, Artandi MK, Oro AE, Artandi SE. 2005. Conditional telomerase induction causes proliferation of hair follicle stem cells. Nature 436:1048–1052.

Stewart SA, Hahn WC, O'Connor BF, Banner EN, Lundberg AS, Modha P, Mizuno H, Brooks MW, Fleming M, Zimonjic DB, Popescu NC, Weinberg RA. 2002. Telomerase contributes to tumorigenesis by a telomere length-independent mechanism. Proc Natl Acad Sci USA 99:12606–12611.

Wei W, Clarke CJ, Somers GR, Cresswell KS, Loveland KA, Trapani JA, Johnstone RW. 2003. Expression of IFI 16 in epithelial cells and lymphoid tissues. Histochem Cell Biol 119:45–54.

Weinrich SL, Pruzan R, Ma L, Ouellette M, Tesmer VM, Holt SE, Bodnar AG, Lichtsteiner S, Kim NW, Trager JB, Taylor RD, Carlos R, Andrews WH, Wright WE, Shay JW, Harley CB, Morin GB. 1997. Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT. Nat Genet 17:498–502.

Wen Y, Yan DH, Spohn B, Deng J, Lin SY, Hung MC. 2000. Tumor suppression and sensitization to tumor necrosis factor alpha-induced apoptosis by an interferon-inducible protein, p202, in breast cancer cells. Cancer Res 60:42–46.

Xin H, Curry J, Johnstone RW, Nickoloff BJ, Choubey D. 2003. Role of IFI 16, a member of the interferon-inducible p200-protein family, in prostate epithelial cellular senescence. Oncogene 22:4831–4840.

Xin H, Pereira-Smith OM, Choubey D. 2004. Role of IFI 16 in cellular senescence of human fibroblasts. Oncogene 23:6209–6217.