The cytoskeleton adaptor protein ankyrin-1 is upregulated by p53 following DNA damage and alters cell migration

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Cells respond to DNA damage by orchestrating a series of events, either resulting in cell-cycle arrest and DNA repair, or elimination of the damaged cell. DNA double-strand breaks (DSB) are one of the most toxic types of DNA damage experienced by cells.¹ A complex network of mechanisms has evolved to detect and repair DSBs. DNA repair is achieved either via non-homologous end-joining or in a more accurate manner by homologous recombination.² Failure of either of these mechanisms triggers apoptosis.³

The DNA damage response pathway (DDR) involves a cascade of events with multiple effector components,⁴–⁷ key to which is the tumour suppressor protein p53.⁸ DNA damage leads to stabilisation of p53 resulting from the degradation of its ubiquitin ligase, MDM2.⁹ This induces the transcription of genes whose products trigger cell-cycle arrest, DNA repair or apoptosis.⁷ More recently, p53 has been shown to regulate certain microRNAs (miRNA) by facilitating their transcription or modulating the activity of the miRNA biogenesis machinery.¹⁰

MiRNAs are ~22 nt RNA molecules which base pair with target mRNAs resulting in translation inhibition and destabilisation of the bound mRNA.¹¹ These small RNAs are involved in a range of biological processes and regulate more than half of all protein-coding genes.¹¹ For example, the transcriptional activation of the miR-34 family by p53 following DNA damage¹² results in the inhibition of key targets, including the transcription factor c-Myc which controls genes involved in cell-cycle progression and cell growth.¹³,¹⁴

In addition to its roles in cell death, p53 has also been implicated in cell motility,¹⁵ and mutant p53 promotes tumour cell invasion and results in loss of directionality during migration.¹⁶ Cell migration is a complex process and is controlled by many proteins,¹⁷ and the specific role of p53 in this mechanism is not yet completely understood.

Here, we initially set out to identify new miRNAs associated with DDR. We found miR-486-5p levels increased ~8-fold following DNA damage, and to our surprise, found the host gene ANK1 increased ~80-fold. We show that both miR-486 and ANK1 are regulated by p53 and that miR-486-5p is involved in controlling G1/S transition following DNA damage. On the other hand, ankyrin-1 plays a role in sustaining cell motility through actin cytoskeleton remodelling upon non-apoptotic levels of DNA damage. Importantly, we found that high ANK1 levels correlate with reduced survival in cancer patients.

Results

Identification of miRNAs upregulated following DNA damage. To identify miRNAs that change following DNA damage, we treated the non-tumorigenic MCF10A cell line with the DNA topoisomerase II inhibitor doxorubicin to induce...
DSBs\(^1\) (Figure 1a) and subjected them to small RNA deep sequencing (Figure 1b). Induction of histone H2A.X phosphorylation (\(\gamma\)-H2AX, Ser139) and p53 phosphorylation (Ser15) confirmed the activation of DDR.\(^4\) The dose and duration of damage were chosen to avoid apoptotic induction, confirmed by the absence of PARP cleavage (Figure 1a and Supplementary Figure S1).

Analysis revealed six miRNAs that showed significant upregulation following damage (\(P<0.05\)), all of which showed >2-fold change (Figure 1b). Two miRNAs were downregulated \(\sim 3\)-fold (\(P<0.05\)) (miR-92a-1-5p and miR-509-3p). Among the upregulated miRNAs were members of the miR-34 family, including miR-34c and miR-34a which are known to be induced following DNA damage.\(^{19–21}\) miR-143-3p

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**Figure 1** Identification of microRNAs upregulated following DNA damage. (a) DNA damage was induced in MCF10A cells with doxorubicin (Dox.) at a concentration of 400 nM for 8 h, and was confirmed by western blots for phospho-p53 (Ser15) and \(\gamma\)-H2AX. Absence of cleaved PARP (89 kDa) confirmed cells were not undergoing apoptosis. Representative western blots shown (\(n=3\)). (b) Small RNA deep sequencing (Illumina) was carried out on total RNA from control and DNA damaged MCF10A cells. MicroRNAs with a significant fold-change (\(P<0.05\)) (\(n=3\)) are shown, with normalised reads displayed as reads per million (RPM). (c) Validation of miR-486-5p upregulation following DNA damage in MCF10A cells that had been exposed to doxorubicin (400 nM) for 0–48 h. MiR-486-5p levels were quantified via qPCR, as well as miR-34 family members as a comparison. Values are mean \pm S.D. (\(t\)-test, \(n=3\), compared with 0 h control), *\(P<0.05\), **\(P<0.01\) and ***\(P<0.001\); NS, not significant. Corresponding western blots are shown in Figure 2c.
which has been previously implicated in DDR, and miR-200a which has a well-known role in cancer metastasis and DNA damage checkpoint control. Importantly, we also found that miR-486-5p increased by 3.8-fold following DNA damage and was relatively abundant, suggesting a possible functional role in DDR. MiR-486 is a muscle-enriched miRNA, but relatively little is known about its involvement in DDR.

To validate these results and determine the temporal nature of miR-486-5p induction, we performed a doxorubicin time-course using MCF10A cells. MiR-34a and miR-34c were used as positive controls (Figure 1c). As expected, miR-34a increased 5.4-fold after 24 h of doxorubicin treatment and 8.0-fold after 48 h. MiR-34c increased 21.5-fold after 24 h of doxorubicin treatment and remained high at 48 h. A significant 1.5-fold increase in miR-486-5p was detectable as early as 4 h following doxorubicin exposure, and increased to 8.3-fold after 48 h. We therefore selected miR-486 for further investigation.

The miR-486 host gene ANK1 is also induced following DNA damage. Approximately half of the 2588 miRNAs in the human genome are intragenic, and there is often a functional relationship between the miRNA and its host gene. Intragenic miRNAs can be regulated either by the host gene’s promoter or an independent promoter. MiR-486 is located within the last intron of the cytoskeleton adaptor gene ANK1 (Figure 2a). Therefore we asked if the primary host gene transcript, ANK1, was also induced following DNA damage. It was previously shown that miR-486 is controlled by a p53 promoter located 21.4 kb upstream of ANK1, but to our knowledge no one had previously examined the role of ANK1 in relation to DNA damage, miR-486 or activation of the p53 pathway.

Strikingly, we found that ANK1 mRNA was upregulated 16-fold after 8 h of doxorubicin-induced DNA damage (Figure 2b) and 110-fold after 16 h, which was markedly higher than the increase in miR-486-5p expression (Figure 1c). To compare this with a well-known DNA damage-induced transcript, we measured mRNA levels of the p53-regulated gene p21Waf1/Cip1, which showed a 25-fold increase following 16 h of doxorubicin treatment (Figure 2b). The dramatic induction of ANK1 mRNA expression levels led us to investigate whether the ankyrin-1 protein was similarly induced. Indeed, we observed that the 246 kDa ankyrin-1 protein was induced ~31-fold after 16 h of doxorubicin treatment (Figure 2c), and ~76-fold after 48 h. Importantly, this was restricted to the canonical ankyrin-1 protein, as the shorter, muscle-enriched isoform of ankyrin-1 (sAnk1) only increased by ~2-fold after 24 h of treatment (Figure 2c).

Next we examined if ankyrin-1 levels increased following exposure to other DNA damage inducers. We found that ionising radiation (IR) exposure induced ankyrin-1 expression in MCF10A cells (Figure 2d), despite low levels of the DNA damage marker γ-H2AX. Ankyrin-1 levels were also induced by the DNA crosslinking reagents cisplatin and mitomycin C (Figure 2d), and also by a different DNA topoisomerase II inhibitor, etoposide (Figure 2c). Ultraviolet-C irradiation did not markedly induce p53 phosphorylation or ANK1 expression, although high levels of γ-H2AX were observed (Figure 2d).

To examine if the upregulation of ankyrin-1 was specific to MCF10A cells, we treated another non-tumorigenic cell line, retinal pigment epithelium (RPE) cells, and also breast cancer-derived MCF7 cells with etoposide. Both RPE and MCF7 cells showed a marked increase of ankyrin-1 protein levels following DNA damage. Ankyrin-1 was also induced by IR exposure in A549 lung carcinoma cells (Figure 2e). Taken together, these data demonstrated that ANK1 was induced in response to various DNA damaging agents in different cell lines, suggesting an important role for ANK1 in DDR activation.

ANK1 and miR-486 expression are p53 dependent. To investigate if the transcription factor p53 was co-regulating both miR-486 and ANK1 following DNA damage, we depleted p53 with siRNA prior to doxorubicin treatment (Figure 3a). In control cells, DNA damage induced miR-486-5p expression by 11.6-fold and ANK1 expression by 26.5-fold. However, knockdown of p53 impaired the induction of both miR-486-5p and ANK1 (Figure 3a).

We then used p53-null cells that harbour either a doxycycline-inducible wild-type p53 or dominant-negative mutant p53R175H construct (Figure 3b). Upon induction of wild-type p53 expression with doxycycline, a 3.7-fold induction of miR-486-5p and a 4.9-fold increase in ANK1 mRNA were observed. Importantly, ankyrin-1 was also induced at the protein level (Figure 3b). As expected, expression of mutant p53R175H did not induce p21Waf1/Cip1. Likewise, both miR-486-5p and ANK1 were not induced by mutant p53, demonstrating that only wild-type p53 could induce their expression.

Finally, we asked whether MCF10A cells treated with the p53 activator nutlin could induce ANK1 expression independently of DNA damage. In control cells ankyrin-1 was barely detectable (Figure 3c); however, its expression was induced following treatment with etoposide or nutlin. The stabilisation of p53 by nutlin was confirmed by MDM2 and p53 western analysis (Figure 3c). Taken together, these data strongly suggest that both miR-486 and ANK1 are induced following DNA damage in a p53-dependent manner.

miR-486-5p inhibition reverses DNA damage-induced cell-cycle arrest. Previously it has been reported that miR-486 expression can inhibit cell growth resulting in cell-cycle arrest in G1. However, these publications did not examine the role of miR-486 following DNA damage in conjunction with the role of the host gene ANK1 in cell-cycle control. To clarify this, we first set out to establish the individual contributions that increased ankyrin-1 or miR-486-5p levels had on cells following DNA damage. We used siRNA to deplete ANK1 and anti-miRs to inhibit miR-486-5p (Figures 4a and b). We confirmed that siRNA depletion of ANK1 resulted in the loss of ankyrin-1 induction following DNA damage (Figure 4a), but did not affect the induction of miR-486-5p (Figure 4b). Likewise, the inhibition of miR-486-5p resulted in ablation of the miRNA induction post-DNA damage (Figure 4b), but did not significantly affect the induction of ankyrin-1 protein (Figure 4a). Now that we could separate the contributions of the miRNA and miRNA...
components, we examined the individual effects of \textit{ANK1} and miR-486-5p on cell-cycle arrest following DNA damage (Figure 4c). We used etoposide treatment for the fluorescence-activated cell sorting (FACS) experiments because doxorubicin and propidium iodide have overlapping fluorescence emission wavelengths. In control siRNA-transfected cells treated with etoposide, we observed a significant decrease in the G1 population, with a corresponding increase in G2, implying G2/M arrest. Activation of DDR was confirmed by detection of phospho-ATM, phospho-p53 and \(\gamma\)-H2A.X (Figure 4a). The depletion of ankyrin-1 following DNA damage (Figure 4a) did not have any effect on the proportion of cells arrested in G2 (Figure 4c). This suggests that DNA damage-induced G2 arrest was
ANK1-independent. Importantly, the inhibition of miR-486-5p in cells treated with etoposide resulted in a reduction of cells in G2/M (Figure 4c).

M-phase arrest induced after nocodazole treatment was used to confirm that the increase in the G1/S population is not caused by cells escaping G2/M arrest and re-entering the cell cycle. Nocodazole-treated cells had a higher proportion of cells remaining in G1 following miR-486-5p inhibition, with or without DNA damage (Supplementary Figure S2). This suggests that miR-486 contributes to G1/S cell-cycle checkpoint control.

Ankyrin proteins are composed of three parts: an N-terminal membrane-binding region containing multiple ankyrin repeat domains, a central spectrin-binding region and a C-terminal regulatory region. Examination of the ankyrin-1 protein sequence revealed a death domain within the C-terminus (Supplementary Figure S3). Death domains are known to interact with components of the apoptosis pathway.

Figure 3  ANK1 and miR-486 expression are p53 dependent. (a) MCF10A cells were subjected to DNA damage using 400 nM doxorubicin (dox.) for 24 h following 48 h siRNA-mediated depletion of p53 or a control siRNA. ANK1 and miR-486-5p RNA levels were determined by qPCR and representative western blots show ankyrin-1 (ANK1), γ-H2AX and phospho-p53 (Ser15) levels following DNA damage and p53 depletion (n = 3). (b) Doxycycline inducible wild-type p53 or mutant p53R175H H1299 cells were either treated (induced) or not treated with doxycycline. Wild type or mutant p53 expression was confirmed via western blot of p53 and p21 (Waf1/ Cip1), and ANK1 and miR-486-5p RNA levels were determined by qPCR (n = 3). (c) Cells were treated with the p53 activator, nutlin. Total p53 and ankyrin-1 levels were analysed by western blot and compared with DNA damage-induced cells treated with the topoisomerase II inhibitor etoposide (etop.) (25 μM) for 24 h. Representative western blots are shown (n = 3). For bar charts, values are mean ± S.D. (t-test, n = 3), *P < 0.05, **P < 0.01 and ***P < 0.001.
pathway. We therefore examined if the induction of ankyrin-1 protein could induce apoptosis. We did not observe any cleaved caspase-3 following either induction or siRNA depletion of ANK1 (Figure 4a). Similarly, miR-486 expression in the presence or absence of etoposide treatment did not induce caspase-3 activation despite previous work, implicating miR-486 in augmenting DDR. To investigate further, we conducted caspase-3/7 activity assays using cell lysates and annexin V-FITC staining of live cells, but failed to observe any increase in caspase activity or apoptosis (data not shown).

Taken together, these data suggest miR-486-5p is involved in maintaining cell-cycle arrest following DNA damage. However, increased ankyrin-1 levels do not appear to affect regulation of the cell-cycle or apoptosis in damaged cells. Owing to the marked induction of ankyrin-1 protein following DNA damage, we decided to further explore other functions of ankyrin-1 following DNA damage.

Ankyrin-1 depletion inhibits cell migration and affects the formation of actin-rich plasma membrane protrusions following DNA damage. Upon DNA damage, cell migration is decreased but is actively maintained through ATM signalling. Ankyrin-1 functions as a membrane adaptor protein, connecting cell membrane proteins to the plasma membrane.
spectrin-actin cytoskeleton, thus implicating it in cell migration. Moreover, it is well-known that p53 can inhibit cell motility through regulation of cell morphology, cell spreading and Rho signalling. Given that ANK1 is controlled by the p53 pathway, we investigated whether ANK1 plays a role in modulating cell motility. To this end, we performed wound-healing assays on cells treated with either control or ANK1 siRNA, with or without etoposide (Figure 5a). Cells that had not been exposed to DNA damage underwent wound closure at the same rate in the presence or absence of ANK1 siRNA (Figures 5b–d). As expected, cells treated with etoposide did not achieve full wound closure (Figures 5b–d). Surprisingly, cells that lacked ankyrin-1 were even more severely affected upon etoposide treatment. For

Figure 5 Ankyrin-1 depletion inhibits cell migration following DNA damage. (a) siRNA was used to deplete ANK1, or cells were treated with a negative control siRNA. Following siRNA treatment, DNA damage was induced with etoposide (etop.) (25 μM) for 24 h. Representative western blots are shown (n=3). (b) Wound healing assays (scratch assay) was performed on confluent MCF10A cells and migration into the wound was monitored for 48 h using time-lapse microscopy. Values are mean ± S.D. (t-test, n=3), *P<0.05. (c) Representative images of MCF10A scratch assays at the initial time of scratch (0 h) and after 24 and 48 h (n=3). Scale bar represents 200 μm. (d) Representative cell tracking assays taken from 10 randomly selected cells from each condition undergoing wound healing.
example, at 36 h after wound generation, ANK1-expressing cells treated with etoposide achieved 68.6% scratch wound closure compared with 38.3% in the absence of ankyrin-1 (Figure 5b). Ankyrin-1 depletion also impaired cell motility in a transwell migration assay (Supplementary Figure S4). These data suggested that ankyrin-1 has a role in sustaining cell movement following DNA damage. Importantly, videos of wound healing assays (Supplementary Movies) confirmed impaired movement of ANK1-depleted cells following DNA damage, showing lamellipodia ruffling which is indicative of inhibited actin filament turnover.42 Time-lapse videos also revealed that DNA damaged cells depleted of ankyrin-1 displayed rapid extension and retraction of membrane protrusions with very little movement of the cell body. The lack of directional movement of these cells was demonstrated by cell tracking assays, which depict the movement patterns of individual cells (Figure 5d).

Intrigued by these observations, we proceeded to examine the cytoskeleton, specifically looking at the morphology of actin filaments in response to DNA damage in the presence or absence of ankyrin-1. Interestingly, it was observed that control cells treated with etoposide had longer, more prevalent actin-rich plasma membrane protrusions compared with etoposide-treated cells following ankyrin-1 depletion (Figure 6a). Quantification revealed that the removal of ANK1 by siRNA decreased the proportion of cells with long

Figure 6 Expression of ANK1 following DNA damage affects actin-rich plasma membrane protrusions. (a) Representative immunofluorescent confocal images of cells treated with either control or ANK1-specific siRNAs, followed by etoposide (etop.) (25 μM) treatment for 24 h to induce DNA damage (n = 3). Actin was stained using Alexa Fluor 488 phalloidin (green) and nuclei were stained using DAPI (blue). White arrows point to actin-rich plasma membrane protrusions. Scale bar represents 50 μm. (b) Long actin processes protruding from cells (examples indicated in (a) with white arrows) were measured on 80 randomly selected cells from each condition. Only cells that were not in colonies were recorded. Measurements were taken from three independent repeat (rep.) experiments and actin protrusion length from the cell membrane was plotted by size (longest to shortest) for each condition in each repeat experiment, with cells lacking any protrusions being represented by a blank space (a length of zero). (c) Percentage of cells with actin protrusions that were greater than 50 units (arbitrary) from each condition. Values are mean ± S.D. (t-test, n = 3). **P < 0.01. (d) Representative western blots (n = 3) of proteins from cells used in confocal microscopy, showing ankyrin-1 (ANK-1) levels following DNA damage, with or without siRNA depletion.
Actin protrusions following DNA damage from 27.1% to 8.8% (Figures 6b–d). These data suggested that ankyrin-1 may play a role in regulating actin cytoskeleton structure following DNA damage.

Actin remodelling factors are modulated by ankyrin-1. As ankyrin-1 appeared to affect actin filament structure and cell motility, we set out to identify how upregulation of ankyrin-1 regulates actin remodelling. It is possible that increased ankyrin-1 levels alone could inhibit actin filament organisation through increased spectrin-actin binding or, alternatively, ankyrin-1 could act to modulate actin remodelling signalling pathways.

Since ankyrin repeats (ANK) have been shown to facilitate nuclear import for some proteins, we first investigated the localisation of ankyrin-1 protein following DNA damage. We found that the ankyrin-1 protein was present predominantly in the cytoplasmic compartment (Figure 7a). We then investigated the Rho GTPase signalling pathway, which controls the remodelling of the actin cytoskeleton. To ensure the changes in this pathway were specific to increased levels of ankyrin-1 and not due to other events triggered by DNA damage, we overexpressed ANK1 in MCF10A cells (Figure 7b). We observed a strong reduction in PAK1 levels and also a reduction in the phosphorylation of the actin-severing protein cofilin (Figures 7b and c). Both of these proteins are key regulators of actin remodelling. Examination of the tumour suppressor p27Kip1, which has recently been implicated in cell migration by regulating RhoA, revealed that it too was significantly reduced upon ANK1 overexpression (Figures 7b and c).

The impact of ANK1 expression in cancer patients. To understand the potential clinical relevance of ANK1 expression levels, we analysed gene expression data from cancer patients using a database tool. We divided the patient cohort into two groups: the first, where a positive correlation between TP53 and ANK1 expression is present, and the second, where it is absent or negative. This analysis allowed us to ask if the link between TP53 and ANK1...
was significant in a clinical setting. Importantly, we found that patients with a high positive correlation between TP53 and ANK1 showed increased survival (Supplementary Figure S5).

Having established the relationship between TP53 and ANK1 in the clinical setting, we next asked how ANK1 expression alone relates to patient survival. Interestingly, we found that high ANK1 expression correlated with decreased survival in lung cancer, breast cancer and CLL (Figure 8a). Since we have demonstrated ANK1 plays a role in cell motility following DNA damage (Figures 5 and 6), we hypothesised that high levels of ANK1 could accelerate the metastasis of cancer cells that are resistant to chemotherapy. To examine this we looked at the relationship between ANK1 and genes associated with DNA repair, such as ATM and CHEK1. Indeed, we identified that high levels of both ANK1 and either ATM or CHEK1 have a negative additive effect on survival (Figure 8b). These data suggest that ANK1 is connected with the DDR and its expression has clinical significance.

Discussion

Previous work investigating the function of miR-486 demonstrated roles in skeletal muscle development, regulation of NF-κB signalling and the modulation of phosphoinositide-3-kinase (PI3K)/Akt signalling. In this study, we have shown that both miR-486 and ANK1 are co-regulated by p53 and are both induced following DNA damage. Previously, miR-486 had been shown to be DDR induced and p53 regulated. However, these studies overlooked the induction of its host gene, ANK1. Our data show that ANK1 is induced to a greater extent than the embedded miRNA following DNA damage. Genome-wide ChIP analysis for p53-binding sites has identified a p53-binding motif upstream of ANK1.

MiR-486 has been shown to be controlled by the transcription factor MRTF-A at a promoter immediately upstream of exon 39a within the ANK1 gene, which also allows for the muscle-specific production of s Ank1 (ANK1:5,57,58). Concurrently, miR-486 has also been shown to be regulated by a p53 promoter upstream of the ANK1 gene in lung cancer. Our work shows that p53 controls both miR-486 and full-length ANK1 expression following DNA damage. It is known that the ANK1 gene is regulated by erythroid transcription factor GATA-1 and transcription factor NF-E2, but its expression in relation to p53 has never been examined. It is not unreasonable to assume that the same p53-binding site located 21.4 kb upstream of ANK1, previously shown to regulate miR-486, may control the expression of both transcripts. However, we cannot rule out the possible usage of other uncharacterised p53-binding sites.

Previous reports demonstrated that the overexpression of miR-486 results in inhibition of cell growth and blocks cell-cycle progression, but the effect of miR-486 on cell-cycle arrest following DNA damage has not been examined. We investigated this using non-tumorigenic MCF10A cells that arrest at G2/M following DNA damage, and observed that inhibition of miR-486-5p resulted in a reduction of cells in G2/M. This suggests that, similar to the miR-34 family, miR-486-5p may play a role in the maintenance of cell-cycle arrest following DNA damage. Further investigation is required to identify mRNA targets of miR-486-5p, the repression of which would facilitate cell-cycle arrest during DDR.

Intriguingly, whereas miR-486-5p leads to the inhibition of cell-cycle progression, ankyrin-1 appears to play no role in cell-cycle maintenance, but rather has an effect on actin remodelling and facilitating cell motility. Indeed, the ankyrin protein has been implicated in cell migration. Ankyrins are highly conserved adaptor proteins that connect a variety of membrane-spanning proteins with the spectrin-actin cytoskeleton. As ankyrin-1 has been shown to bind to TIAM1, which in turn stimulates GDP/GTP exchange activity on Rac1, we looked at a panel of molecular components in the actin remodelling pathway. We found that overexpression of ANK1 severely hampered the expression of PAK1, p27kip1 and phospho-cofilin levels, but had little effect on levels of ROCK1/ROCK2 or TIAM1. It is known that cofilin, along with ARP2/3 and profilin, is one of the main effectors of actin remodelling. Therefore, we speculate that the lack of actin protrusions and perturbed migratory behaviour in DNA damaged cells lacking ankyrin-1 may be attributed to ankyrin-1 levels modulating components of the Rho GTPase signalling pathway. This ultimately results in impaired cell motility. The physiological reason for the apparent facilitation of cell migration as a result of ANK1 upregulation following DNA damage is currently unclear; however, it is in line with other previously documented observations.

In summary, we have shown how p53 can regulate a single transcript that produces two distinct molecular units with very different functions: a structural protein to facilitate motility and a co-expressed miRNA to attenuate cell-cycle progression following DNA damage. We also implicate a novel role for ankyrin-1 in the DDR. In line with our findings, a lack of TP53/ANK1 correlation predicts a poor patient outcome in clinical data sets. Importantly, we found that higher ANK1 levels alone correlated with lower tumour-free patient survival in the clinical setting. Moreover, high expression levels of ANK1 and DNA repair genes, such as ATM and CHEK1, in combination, are additive in predicting negative outcome. In light of the role ANK1 plays in cell motility post-DNA damage, we postulate that high levels of ANK1 could accelerate the metastasis of cancer cells that are resistant to chemotherapy. This suggests that ANK1 expression might potentially be used as a prognostic marker in patients with specific tumours.

Materials and Methods

Cell culture. MCF10A cells were cultured in DMEM/F12 (Gibco, Grand Island, NY, USA) with 5% horse serum, 1% glutamine, 20 ng/ml recombinant human EGF (Peprotech, Rocky Hill, NJ, USA), 10 µg/ml insulin, 500 ng/ml hydrocortisone and 10 ng/ml chola toxin. MCF7 and A549 cells were cultured in DMEM with 10% fetal bovine serum (FBS) and 1% l-glutamine. The p53/R175H doxycycline-inducible H1299 cells were described previously and were cultured in DMEM containing 10% tetraacycline-free FBS and 1% glutamine. Gene expression of p53/R175H was induced by culturing cells in 2 µg/ml doxycycline for 48 h. RPE cells were cultured in DMEM/F12 with 10% FBS, 1% glutamine and 0.16% sodium bicarbonate. All cells were grown at 37 °C in a 5% CO2 humidified incubator.

DNA damage and p53 induction. DNA damage was achieved using either 25 µM etoposide (24 h) (Cayman, Ann Arbor, MI, USA) in dimethyl sulphoxide (DMSO), 400 nM doxorubicin hydrochloride (0–48 h) (Sigma, St. Louis, MO, USA), 10 µg/ml cisplatin (24 h) (Sigma) or 300 nM mitomycin C (24 h) (Sigma). IR was produced by an Xstrahl X-Ray irradiator: Xstrahl (Camberley, UK) and ultraviolet
Figure 8  ANK1 expression in cancer patient survival. (a) Kaplan–Meier survival plots of patients with lung cancer (GEO data set: GSE31210), breast cancer (GEO data set: GSE25055) and chronic lymphocytic leukaemia (GEO data set: GSE22762) with either high (red) or low (green) ANK1 expression. Data obtained using the PPISURV database tool using different Gene Expression Omnibus (GEO) data sets. (b) Correlation between expression of DNA damage response regulators (ATM and CHEK1) and survival in breast cancer patients (GEO data set GSE25066), and the synergistic effect of high ANK1 levels. High expression of genes shown in red or low in blue. Data obtained using SynTarget bioinformatics analysis tool (www.bioprofiling.de)
light was produced using a UV crosslinker CX-2000 (UVP, Upland, CA, USA). The p53 activator nutilin-3 (Sigma) was prepared in DMSO and used at 10 μM for 24 h.

**Western blotting and antibodies.** Cells were harvested in RIPA buffer (150 mM NaCl) and lysates were sonicated for 2.5 min prior to clarification. Total protein (20–40 μg) was separated on polyacrylamide gels and transferred onto nitrocellulose membrane, 0.45 μM. All antibodies were used at dilutions recommended by the manufacturer. Antibodies used were: ANK1 (Novus Biologicals, Littleton, CO, USA, NBP1-71805) – for detection of endogenous protein, ANK1 (Atlas Antibodies, Stockholm, Sweden, HP A056953) – for detection of cloned protein, sAnk1 (Abcam, Cambridge, UK, ab58698) – for detection of the small isoform, Total p53 (Santa Cruz, Dallas, TX, USA, sc-126), Phospho-p53 (Ser15) (Cell Signalling, Danvers, MA, USA, #9284), MDM2 (Santa Cruz, sc-965), Total H2A.X (Cell Signalling, #2595), Phospho-histone (γ) H2AX (Ser139) (Cell Signalling, #9718), PARP (Cell Signalling, #9542), B-actin (Sigma, #A5441), p21WAF1/CIP1 (Santa Cruz, sc-397), Phospho-ATM (Ser1981) (Abcam, ab1292), Caspase-3 (Cell Signalling, #9662), GAPDH (Santa Cruz, sc-25233), eIF4G (Cell Signalling, #2498), Lamin A/C (Santa Cruz, sc-27832), p27 (Cell Signalling, #33660), PAK1 (Bethyl Laboratories, Montgomery, TX, USA, A301-295T), Total cofilin (Cell Signalling, #5175), Phospho-cofilin (Ser3) (Cell Signalling, #3313), ROCK2 (Bethyl Laboratories, A300-047A-T), TIM1 (Bethyl Laboratories, A300-097AT) and Vinculin (Abcam, ab18058). IR-Dye-labelled secondary antibodies (LI-COR) were used to detect primary antibodies. Blots were scanned with a LI-COR laser-based image detector: LI-COR Biosciences, (Lincoln, NE, USA) and analysed using Image Studio software (v.2.1).

**Small RNA library preparation.** Total RNA was purified using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Small RNA libraries were prepared using the TruSeq Small RNA kit (Illumina, San Diego, CA, USA) with 0.5 μg total RNA. Libraries were sent to the Genome Analysis Centre (TGAC), Norwich. The small RNA libraries were checked on a BioAnalyser (Agilent DNA 2100 chip, Agilent, Santa Clara, CA, USA) and LabChip GX, followed by quantification. Libraries were pooled and sequenced on the Illumina HiSeq 2500 (Rapid-Run mode, cBot platform) using 50 bp single end reads.

**Bioinformatics analysis of deep sequencing data and accession numbers.** All sequencing files were transformed from FASTQ to FASTA format and the adaptors were removed by matching the first 8 nt of adaptor sequence (TTGAATTTC). These reads were then mapped to mature human miRNA sequences from miRBase version 25

**Quantitative PCR and TaqMan assays.** MicroRNAs were quantified using TaqMan assays (Applied Biosystems, Foster City, CA, USA) with 100 ng input total RNA. MicroRNA and sRNA-specific reverse transcription primer and probe reagents were used in a 20 μl reaction volume: has-miR-486-5p (H00278), has-miR-34a-5p (H000426), has-miR-34c-5p (H000428) and U6 snRNA (H001973). SYBR green (Applied Biosystems) based quantitative PCR (qPCR) was used for mRNA quantification. miR-34c-5p (#000428) and U6 snRNA (#001973). SYBR green (Applied Biosystems) based quantitative PCR (qPCR) was used for mRNA quantification. The ΔΔCT method was used to calculate fold-change between the control sample and the other conditions using U6 snRNA as an internal control gene for microRNAs and GAPDH for mRNAs.

**Statistical analysis.** Where stated, statistical significance was analysed using the Student’s t-test (two sample, two tailed, unpaired) and expressed as a P-value. In all bar charts and graphs, error bars represent the standard deviation of relative values/ fold-changes from three experiments. Statistical significance for cancer patient data analysis was calculated using the relevant bioinformatics tool (see below).
Determination of p53/ANK1 correlation and survival outcome in cancer patients. In contrast to the standard approach, where patients are grouped based on high/low expression of a gene, in this case, patients are grouped based on presence/absence of correlation between p53/ANK1 and the expression of the other genes on survival of cancer patients. Patients in the clinical data sets are split into four groups with respect to the expression of the ANK1 and CHK1/ATM genes (high–high, low–low, high–low, and low–high). Next, each group is tested versus other samples to find any statistical differences in survival outcome (i.e. high–high versus others, low–low versus others and so on).

Conflict of Interest
The authors declare no conflict of interest.

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Author contributions
The project was conceived and experiments were planned by AHE and MB. All experiments were conducted by AHE except the ionising radiation experiments in vivo. The project was conceived and experiments were planned by AEH and MB. All aspects of the study were supervised by MB.

The manuscript was prepared by AHE, W-TL, AW and MB and all authors read and approved it.

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