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LETTERS

The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor

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Some stimulatory receptors of the innate immune system, such as the NKG2D receptor (also called KLRK1) expressed by natural killer cells and activated CD8$^+$T cells, recognize self-molecules that are upregulated in diseased cells by poorly understood mechanisms[1]. Here we show that mouse and human NKG2D ligands are upregulated in non-tumour cell lines by genotoxic stress and stalled DNA replication, conditions known to activate a major DNA damage checkpoint pathway initiated by ATM (ataxia telangiectasia, mutated) or ATR (ATM- and Rad3-related) protein kinases[2]. Ligand upregulation was prevented by pharmacological or genetic inhibition of ATR, ATM or Chk1 (a downstream transducer kinase in the pathway). Furthermore, constitutive ligand expression by a tumour cell line was inhibited by targeting short interfering RNA to ATM, suggesting that ligand expression in established tumour cells, which often harbour genomic irregularities, may be due to chronic activation of the DNA damage response pathway. Thus, the DNA damage response, previously shown to arrest the cell cycle and enhance DNA repair functions, or to trigger apoptosis, may also participate in alerting the immune system to the presence of potentially dangerous cells.

To investigate mechanisms leading to NKG2D ligand upregulation, we examined two transformed ovarian epithelial cell lines from *p53*$^{-/-}$ mice[3]. The C1 cell line had been transduced with *K-ras* and c-*myc*, whereas the C2 cell line had been transduced with *Akt* and c-*myc* (Fig. 1a). Both transformed cell lines grew well in cell culture but did not express appreciable levels of mouse NKG2D ligands, as detected by staining with a tetrameric NKG2D reagent that binds to all mouse NKG2D ligands (Rae1, MULT1 and H60 (ref. 1)) (Fig. 1b). Ligand upregulation failed to occur when C1 and C2 cells were transfected or super-transduced with numerous other oncogenes, including *E6*, *E7*, *E1A* or *Ras* V12 (data not shown), some of which interfere with expression of the retinoblastoma tumour suppressor gene. When injected into the ovaries of nude mice, both cell lines generated ovarian epithelial tumours, which were established as cell lines T1 and T2 (ref. 3). Both T1 and T2 exhibited significant upregulation of NKG2D ligands (Fig. 1B), including Rae1 (see below). These findings suggested that ligand upregulation was not associated with transformation *per se*.

Ligand expression by C1 or C2 cells was not upregulated by numerous cell stress conditions, including heat shock, hyperoxia, hypoxia, inhibition of the cell cycle (by roscovitine), exposure to inflammatory cytokines such as tumour necrosis factor, interferon or interleukin (IL)-6, or incubation in medium of pH 6 or pH 8.5 (Fig. 2a, Supplementary Fig. S1 and data not shown). In contrast, NKG2D ligands were upregulated in C1 or C2 cells exposed to high doses of ionizing radiation, inhibitors of DNA replication such as mitomycin C, hydroxyurea, 5-fluorouracil (5-FU) and the DNA polymerase inhibitor aphidicolin, or chromatin-modifying treatments such as trichostatin A, chloroquine and hypotonic conditions[4] (Fig. 2b; see also Supplementary Fig. S1). These same treatments induced ligand upregulation in cultures of adult fibroblasts, showing that neither transformation nor *p53* deficiency are essential for ligand upregulation (Fig. 2b). In the case of fibroblasts, but less so with the C1 and C2 cell lines, ligand upregulation was also induced by other DNA damaging conditions such as ultraviolet light

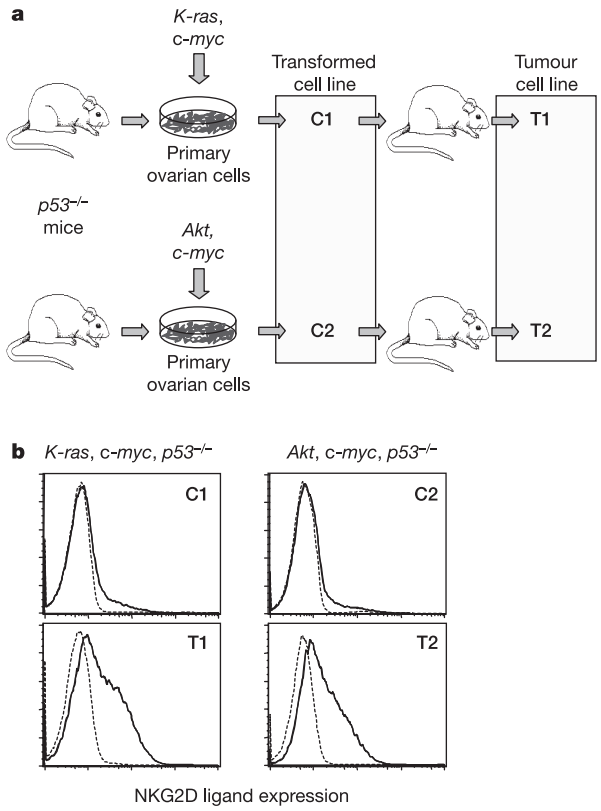

Figure 1 | NKG2D ligand upregulation is associated with tumorigenesis, not with transformation *per se*. **a**, Cell lines from transgenic mice expressing the avian retrovirus receptor. Ovarian epithelial cells from *p53*$^{-/-}$ mice were transduced with the indicated genes and established as cell lines C1 and C2. Implantation of C1 and C2 cells into ovaries of nude mice led to formation of tumours, which were established as cell lines T1 and T2. **b**, Transformed ovarian epithelial cell lines C1 and C2, and T1 and T2 tumour cell lines, were stained with NKG2D tetramers (solid line) or with control tetramers (dashed line).

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and the chemotherapy agents cisplatin and ara-C (Fig. 2b; see also Supplementary Fig. S1). Cell surface ligand upregulation detected with NKG2D tetramers or Rae1 antibodies (Fig. 2b) was accompanied by increases in both Rae1 and MULT-1 messenger RNAs (Fig. 3a; see also Supplementary Fig. S2). Both the mRNA and protein levels were initially detected at 3–5 h, and reached a plateau after 16–24 h (Fig. 3a, b). Ligand levels reverted to background levels 72 h after aphidicolin was washed out of the medium (data not shown).

Similarly, the homologous human NKG2D ligands ULBP1, ULBP2 and ULBP3 were often upregulated in secondary human foreskin fibroblasts treated with high doses of ionizing radiation and inhibitors of DNA replication, including aphidicolin, but not with roscovitine (Fig. 2c). Mitomycin C and aphidicolin also modestly upregulated MICA, another human NKG2D ligand (Fig. 2c and Supplementary Fig. S1).

Mouse T cell blasts treated with aphidicolin also upregulated NKG2D ligands modestly, and exhibited greater sensitivity to lysis by IL-2-activated natural killer (NK) cells (Fig. 2d). Lysis was inhibited by NKG2D antibody, indicating that upregulated ligands induce elevated lysis, but the partial effect suggests that aphidicolin may also induce other NK target ligands.

The treatments that induced ligand upregulation all activate a major DNA damage response pathway initiated by ATR and/or ATM (depending on the treatment). The pathway is mediated by downstream mediators including the Chk1 and Chk2 kinases and p53 (refs 2,5,6), and results in cell cycle arrest, activation of DNA repair pathways, new transcription, and, if damage is extensive, apoptosis. Consistent with a role for this pathway in NKG2D ligand upregulation, phosphorylation of Chk1 on serine 345, which is required for activation of this kinase, occurred before ligand upregulation was detectable within 1 h of treatment of fibroblasts with aphidicolin (Fig. 3c). Phosphorylation of Chk2 on threonine 387 also occurred but was delayed.

The role of ATR in aphidicolin-induced NKG2D ligand expression was investigated with three independent approaches. Ligand upregulation in response to aphidicolin was blocked by caffeine, an inhibitor of ATR and ATM[7], at doses close to the IC_{50} (half-maximal inhibitory concentration) for ATR (1.1 mM, Fig. 4a). In a second approach, ATR gene expression was specifically impaired using siRNAs in adult fibroblast cultures, introduced by transduction with green fluorescent protein (GFP)-expressing retroviruses. Upregulation of NKG2D ligands induced by aphidicolin was strongly inhibited in cells transduced with *Atr* siRNA compared to untransduced (GFP^-) cells in the same cultures or to cells transduced with control siRNA (Fig. 4b). Cells infected with the control siRNA or *Atr* siRNA but not treated with aphidicolin did not upregulate ligands (Fig. 4b). As a third approach, the ATR gene was deleted in cultured cells. ATR function is required for cell viability, necessitating the use of a conditional *Atr* mutation. A fibroblast cell line was derived from adult mice with a gene-targeted *Atr* allele in which two critical exons were flanked by *loxP* sites[8]. Fibroblasts transduced with a Cre recombinase-expressing retrovirus to inactivate the *Atr* gene and treated with aphidicolin showed minimal upregulation of NKG2D ligand cell surface proteins (Fig. 4c), or mRNAs (Fig. 4d), compared with cells transduced with a control murine stem cell virus (MSCV) retrovirus. Control experiments established that both the untreated Cre-transduced Atr^{flox} cells and the untreated MSCV-transduced Atr^{flox} cells proliferated actively (Supplementary Fig. S3), a necessary condition for activation of the replication checkpoint. Proliferation of both cell populations was blocked with aphidicolin (Supplementary Fig. S3). Cre transduction had no effect on aphidicolin-treated wild-type fibroblasts (Fig. 4c, d). Thus, upregulation of NKG2D ligands as a result of replication arrest is *Atr*-dependent.

A role for the Chk1 kinase was suggested by the finding that aphidicolin-induced ligand upregulation was inhibited by three independent Chk1 inhibitors (Fig. 5a). The effective doses were close to the IC_{50} values of all three inhibitors for Chk1 (8 nM staurosporine, 15 nM SB-218078 and 3 μM debromohymenialdisine (DBH)) (Fig. 5a). Each of these inhibitors also acts on targets other than Chk1, but the IC_{50} values differ and the other targets are not all shared by the three inhibitors. As a more specific test, transduction of fibroblasts with *Chk1* siRNA reproducibly inhibited aphidicolin-induced NKG2D ligand upregulation as compared to untransduced

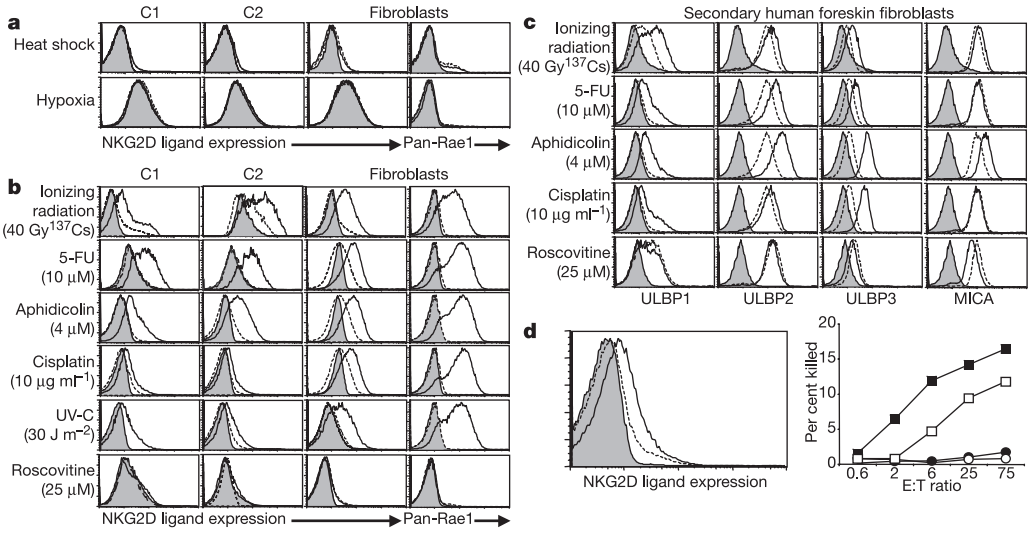

Figure 2 | NKG2D ligands are induced by DNA-damaging agents and DNA synthesis inhibitors. **a**, No induction of ligands in C1 or C2 cells by heat shock (42 °C, 90 min) and hypoxia (1% O_2, 48 h). Cells were stained with NKG2D tetramers or Rae1 antibody. **b**, **c**, Upregulation of ligands in mouse cells (**b**) or human fibroblasts (passage 3–6) (**c**) by DNA-damaging agents and DNA synthesis inhibitors (16 h treatment). Treated cells (solid lines) were compared to untreated cells (dashed lines), or to treated cells stained with control tetramers or isotype control antibodies (filled histograms). **d**, Aphidicolin treatment upregulates NKG2D ligands on T-cell blasts (left panel, key as defined above) and elevates their sensitivity to IL-2-activated NK cells (right). NK cells were incubated with treated (squares) or untreated (circles) target cells in the absence (filled) or presence (open) of NKG2D antibody. The effector to target ratio (E:T) is indicated. In repetitions of the experiment, untreated cells were killed but always substantially less well than treated cells. For all the flow cytometry histograms in **a**–**d**, the *x* axis depicts staining intensity and the *y* axis depicts the relative number of cells.

(GFP$^-$) cells in the same culture or cells transduced with control siRNA (Fig. 5b). The *Chk1* siRNA did not completely abrogate Chk1 protein expression (Fig. 5c), possibly explaining why pharmacological inhibitors were more effective at blocking ligand upregulation. The inhibitor and siRNA results indicate that Chk1 is necessary for optimal NKG2D ligand upregulation in response to aphidicolin treatment.

Ionizing radiation, hypotonic conditions and other treatments that activate ATM, in some cases preferentially to ATR[4,9], also induced upregulation of NKG2D ligands (Fig. 2; see also Supplementary Fig. S1). An ATM siRNA/GFP retrovirus partially inhibited NKG2D ligand upregulation in cells exposed to ionizing radiation or hypotonic conditions, but had no effect on ligand upregulation in cells exposed to aphidicolin or ultraviolet C, which preferentially activate ATR[9] (Supplementary Fig. S4). Ligand upregulation in response to ATM inducers was also inhibited by caffeine, which blocks both ATR and ATM[7] (Supplementary Fig. S4a). Thus, induction of NKG2D ligands requires ATM or ATR, depending on the nature of the treatment.

It is unlikely that NKG2D ligand upregulation is an indirect consequence of ATR/ATM-induced cell cycle arrest, because cell cycle arrest induced by the cyclin-dependent kinase inhibitor roscovitine or serum deprivation did not trigger ligand upregulation (Fig. 2b, c; see also Supplementary Fig. S1). It is also unlikely that ligand upregulation is a property of cells entering the cell death pathway, because such cells, defined by staining with Annexin-V, did not upregulate NKG2D ligands significantly (data not shown). Hence, the DNA damage response probably has a more direct role in the process of ligand upregulation.

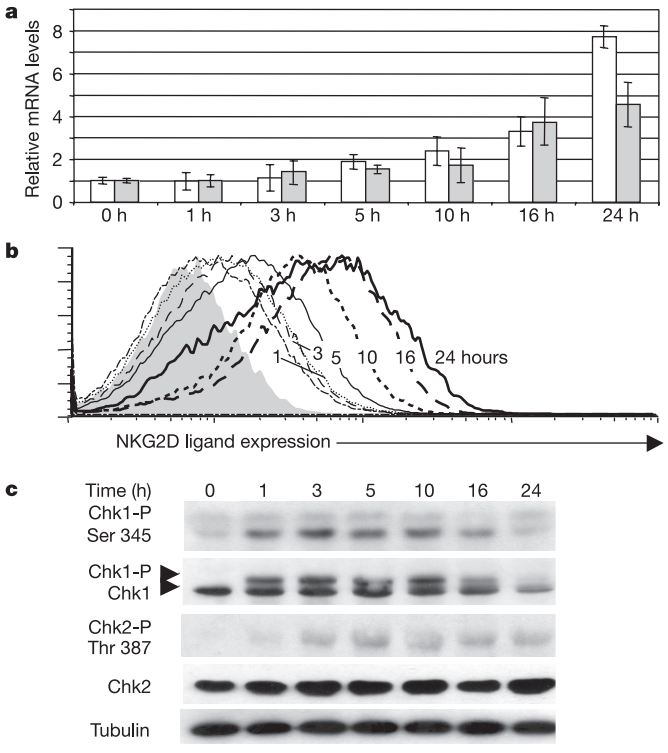

Figure 3 | Kinetics of the upregulation of NKG2D ligands and phosphorylation of the Chk1 kinase. **a**, Levels of Rae1 (open bar) and MULT1 (filled bar) transcripts determined by real-time RT–PCR in cultured fibroblasts treated with aphidicolin for the indicated times. The Rae1 primers amplify all Rae1 isoforms. Data represent means ± s.d., $n = 3$ independent experiments. **b**, Cell surface expression of NKG2D ligands on cells from **a**. Cells treated for the indicated period were compared to untreated cells (dashed and dotted lines), or to 24 h-treated cells stained with control tetramers (fitted histograms). The *y* axis depicts the relative number of cells. **c**, Kinetics of phosphorylation of Chk1 in aphidicolin-treated fibroblasts. Phosphorylation was detected by western blotting as a shift in the mobility of Chk1, or with the use of a phospho-Ser 345 Chk1 specific antibody or phospho-Thr 387 Chk2 specific antibody.

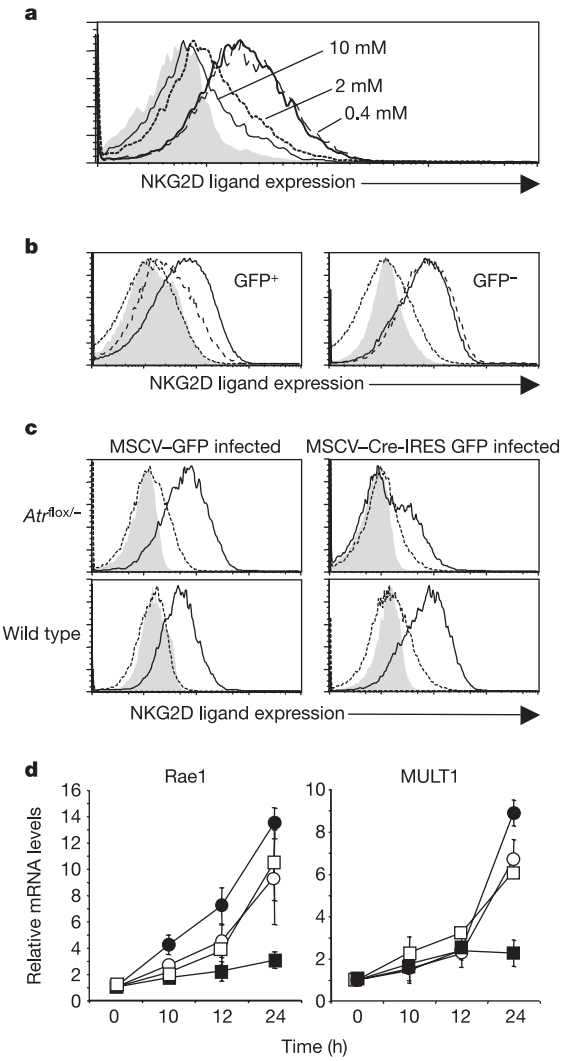

Figure 4 | Ligand upregulation in aphidicolin-treated fibroblasts is dependent on ATR function. **a**, Ligand upregulation inhibited by caffeine (an inhibitor of ATR/ATM) added 1 h before aphidicolin. Aphidicolin-treated cells incubated with the indicated caffeine concentrations were compared to treated cells incubated with no inhibitor (thick, solid line), or to treated cells stained with control tetramers (filled histograms). **b**, Inhibition of ligand upregulation by *Atr* siRNA. Fibroblasts were transduced with retroviral vectors encoding GFP and *Atr* siRNA (dashed lines in histograms) or GFP and control siRNA (solid lines), cultured for 5 days and treated with 4 μM aphidicolin for 16 h. NKG2D ligand expression was determined by gating on transduced (GFP$^+$) and untransduced (GFP$^-$) cells from the same cultures. The filled histogram indicates aphidicolin-treated cells stained with control tetramers; the dotted line indicates untreated cells stained with NKG2D tetramers. **c**, Conditional deletion of the ATR gene prevents ligand upregulation. *Atr*$^{flox/-}$ or wild-type fibroblasts were transduced with MSCV-IRES–GFP or MSCV-Cre-IRES–GFP, cultured and analysed as in **b**, gating on transduced (GFP$^+$) cells. The solid line indicates treated cells stained with NKG2D tetramers; the filled histogram indicates treated cells stained with control tetramers; and the dotted line indicates untreated cells stained with NKG2D tetramers. **d**, Reduced induction of Rae1 and MULT1 transcripts in cells deficient for ATR expression. Real-time PCR of cDNA samples from transduced fibroblast cultures described in **c**. Open symbols, wild type fibroblasts; filled symbols, *Atr*$^{flox/-}$ fibroblasts; circles, control; squares; Cre-transduced. Means ± s.d. of three independent experiments are shown.

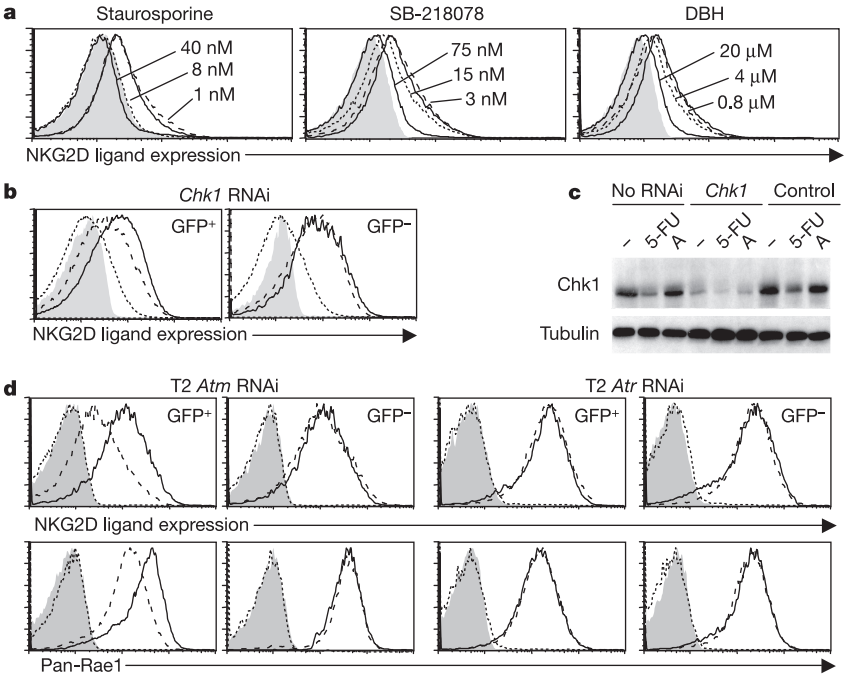

Figure 5 | Role of Chk1 in expression of NKG2D ligands in aphidicolin-treated fibroblasts and tumour cell lines. a, Chk1 inhibitors prevent upregulation of NKG2D ligands. Fibroblasts were pre-incubated with staurosporine, SB-218078, debromohymenialdisine, or DMSO before aphidicolin treatment. The thick, solid line represents treated cells (no inhibitor) stained with NKG2D tetramers; filled histogram represents treated cells (no inhibitor) stained with control tetramers **b**, Inhibition of ligand upregulation by *Chk1* siRNA. Fibroblasts were transduced with retroviral vectors encoding GFP and *Chk1* siRNA (dashed lines in histograms) or GFP and control siRNA (solid lines) before treatment, as in Fig. 4b. The filled histogram represents aphidicolin-treated cells stained with control tetramers; the dotted line indicates untreated cells stained with NKG2D tetramers. **c**, Chk1 protein levels are reduced in cells transduced with *Chk1* siRNA. Untransduced or sorted GFP^+ transduced fibroblasts were cultured for 2 days and treated for 16 h with DMSO (labelled −), 4 μM aphidicolin (labelled A) or 10 μM 5-FU. Lysates were prepared and western blotted with Chk1 antibodies. **d**, *Atm* siRNA, but not *Atr* siRNA, decreases levels of NKG2D ligands in T2 cells. T2 cells were transduced with GFP retroviral vectors encoding control siRNA (solid lines) or either *Atm* or *Atr* siRNA (dashed lines in histograms). After culturing for 7 days, NKG2D ligand expression was determined by gating on transduced (GFP^+) and untransduced (GFP^-) cells from the same cultures. Filled histogram, control siRNA transduced T2 cells stained with control tetramer; dotted line, *Atr* or *Atm* siRNA transduced T2 cells stained with control tetramers. For all the flow cytometry histograms in **a**, **b**, **d**, the *x* axis depicts staining intensity and the *y* axis depicts the relative number of cells.

Activation of the DNA damage pathway may represent a more distinctive feature of diseased cells than other correlates of tumorigenesis or infection. The pathway is activated in cells infected with several viruses[10], raising the possibility that it has a role in ligand upregulation after viral infection[11]. The pathway is also frequently activated in tumour cell lines[12–14], possibly due to the greater genomic instability of these cells as compared to transformed cells[13,14]. Furthermore, recent reports have demonstrated the activation of ATM and other components of the DNA damage response pathway in pre-cancerous lesions, at a stage before genomic instability is apparent[15,16]. We addressed whether chronic activation of the DNA damage pathway contributes to constitutive expression of NKG2D ligands observed in the T2 ovarian tumour cell line (Fig. 1). T2 cells transduced with *Atm* siRNA exhibited reduced NKG2D ligand expression, whereas *Atr* siRNA had no effect (Fig. 5d). Thus, constitutive ligand expression in tumour cell lines may be dependent on ATM activity, at least in some instances. Accelerated loss of genomic stability or other cellular changes that activate the DNA damage response in tumour cells may lead to constitutive ATM activation. Further studies will be necessary to establish the generality of the link between the DNA damage response and NKG2D ligand expression in tumour cells.

Transcriptional regulators including the p53 tumour suppressor, p73 (ref. 17) and p63 are activated by Chk1. p53 is not required for ligand upregulation, which occurred in the $p53^{-/-}$ C1 and C2 cell lines, but p73 or p63 may have redundant or unique roles in ligand upregulation. The p53-independent component of NKG2D ligand upregulation means that loss of *p53* during tumorigenesis should not by itself fully disable the immune-enhancing potential of the cells.

These findings suggest a novel link between the immune response and processes that regulate genome integrity, and may have clinical significance. It is possible that part of the efficacy of some chemotherapies and radiotherapies, most of which activate the DNA damage response[18,19], is due to the induction of NKG2D ligands and consequent enhanced sensitivity of the cell to the immune system. The pathway leading to the upregulation of NKG2D ligands may be a productive target for design of therapeutic agents to enhance the immunogenicity of tumour cells while reducing overall toxicity.

METHODS

Mice, cells and cell treatments. C57BL/6 mice from the Jackson Laboratory were bred and housed as described[20]. Ear- and tail-derived fibroblasts from adult C57BL/6 mice or 129SvEv/C57BL/6-$Atr^{flox/-}$ mice[8,21] were established as described[22]. Unless otherwise specified, the ear fibroblasts were used for the experiments. C1, C2, T1 and T2 mouse ovarian cancer cell lines were generated as described[3] and treated for 16 h unless stated otherwise. Secondary neonatal human dermal foreskin fibroblasts were purchased (Cascade Biologics) and cultured in 106 medium.

Hypotonic swelling was carried out for 16 h in PBS containing 0.45% glucose (w/v) and 2% FBS, with the NaCl concentration adjusted to 50 mM or, as a control, 140 mM. For inhibitor experiments, inhibitors were added to subconfluent cultures beginning 1 h before addition of 4 μM aphidicolin, and ligand expression was determined 16 h later.

Reagents. 5-bromo-2′-deoxyuridine (BrdU), 5-fluorouracil (5-FU), aphidicolin, caffeine, *cis*-diammineplatinum(II) dichloride, cytosine β-D-arabino-furanoside

hydrochloride, mitomycin C, hydroxyurea, staurosporine and *t*-butylhydroquinone were purchased from Sigma. Roscovitine, SB-218078 and debromohymenialdisine were purchased from Calbiochem. NKG2D tetramers and control tetramers were produced as previously described[23]. For staining, we used Pan-Rae1, MICA, ULBP1, ULBP2 and ULBP3 specific monoclonal antibodies (R&D Systems), mouse IgG and F(ab′)2 fragment goat anti-mouse or rat IgG plus IgM coupled to phycoerythrin (Jackson ImmunoResearch Laboratories).

MSCV-Cre constructs and transduction of fibroblasts. *Cre* was subcloned adjacent to the cytomegalovirus promoter in the pMSCV2.2-IRES–GFP proviral vector (gift of W. Sha). Retroviral supernatants were generated as described[24].

siRNA retroviral constructs. *Chk1* siRNA (5′-CAACTTGCTGTGAATAGAAT-3′) corresponded to the mouse counterpart of a published human *Chk1* siRNA[25]. *Atr* siRNA (5′-AGGAAGCAATTCCACATTAAGC-3′) and *Atm* siRNA (5′-GAG GTGGCTCTTATTCTAC-3′) were selected based on Dharmacon's siRNA Design Center algorithm (Dharmacon). The control siRNA (5′-AACAAGTGAAGCAG TCGCAGT-3′) has been described previously[26]. The siRNAs were subcloned in the pMND-Banshee vector (a gift of J. Alberola-Ila), as described previously[27]. The control RNAi-pMND-Banshee, ATR-pMND-Banshee and Chk1-pMND-Banshee plasmids were transiently transfected into 293 T cells using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). 48 h after transfection, 50% confluent cultures of fibroblasts or T2 were transduced as described[24].

As determined by real time RT–PCR in sorted populations, the level of ATR transcripts in *Atr*-siRNA-transduced cells in Fig. 4b was 37 ± 3% of the level in untransduced (GFP^-) cells in the same culture (mean ± s.d., $n = 3$). In Fig. 5d, *Atm* mRNA in *Atm*-siRNA-transduced T2 cells was 53 ± 2% of the level in untransduced (GFP^-) cells in the same culture, whereas *Atr* mRNA in *Atr*-siRNA-transduced cells was 49 ± 5% of the level in untransduced (GFP^-) cells in the same culture. Because of impurities in the sorted populations, the knockdowns may be more efficient than indicated.

Western blotting. Whole-cell extracts were prepared from untreated or treated populations, electrophoresed in 6% or 8% SDS–PAGE gels, and blotted onto nitrocellulose membranes. Antibodies against Chk1 (sc-8408, Santa Cruz Biotechnology), phospho-Chk1-Ser 345, Chk2, phospho-Chk2-Thr 387 (all Cell Signalling Technology), tubulin (CP06, Calbiochem) and horseradish peroxidase-coupled second stage reagents were used to develop the blots (SuperSignal West Pico Stable Solution). Blots were exposed on X-ray film.

Quantitative real-time RT–PCR. Total RNA was isolated using the RNeasy kit (Qiagen). Real-time PCR assays were performed using an Applied Biosystems 5700 sequence detector. Two micrograms of total RNA was reverse transcribed with random hexamers using a Transcriptor First Strand cDNA Synthesis Kit (Roche). Each amplification mixture (25 μl) contained 25 ng of reverse-transcribed RNA, 8 μM forward primer, 8 μM reverse primer and 12.5 μl of iTaq SYBR Green Supermix with ROX (Bio-Rad). PCR thermocycling parameters were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 1 min. All samples were normalized to the signal generated from the housekeeping genes *β-actin* or *Gapdh* (for ATR and ATM levels). SYBR green PCR was performed in triplicate. The following primers were used: β-actin-5′, 5′-TGTTTGAGACCTTCAACACC-3′; β-actin-3′, 5′-TAGGAGCC AGAGCAGTAATC-3′; GAPDH-5′, 5′-GAAGGTCGGTGTGAACGGA-3′; GAPDH-3′, 5′-GTTAGT GGGGTCTCGCTCCT-3′; MULT1-5′,5′-CAATGT CTCTGTCCTCGGAA-3′; MULT1-3′, 5′-CTGAACACGTCTCAGGCACT-3′; Pan-Rae1-5′, 5′-TGGACACTCACAAGACCAATG-3′; Pan-Rae1-3′, 5′-CCCA GGTGGCACTAGGAGT-3′; ATR-5′, 5′-TGCGCTCTGCTAGAGCACGGT-3′; ATR-3′, 5′-AGTGCTGGCTGGCTGTGCTG-3′; ATM-5′, 5′-ATCCAGGCCCTG CAGAATTTGGG-3′; ATM-3′, 5′-CTCCACGCCGCCTGGTAACG-3′. Samples prepared without reverse transcription served as negative control templates.

Cytolysis assay. BALB/c spleen cells were activated for 3 days with 2.5 μg ml^{-1} concanavalin A, washed with 50 mM α-methyl mannoside and recultured with 10 U ml^{-1} IL-2 plus either aphidicolin or DMSO for 18 h before labelling with ^{51}Cr for use as target cells. NK cells were prepared as described[20]. Thirty minutes before adding target cells to initiate the 3-h cytolysis assay[20], antibody MI-6 (anti-NKG2D[20]) was added to a concentration of 50 μg ml^{-1} in some groups. Spontaneous release was 5% for DMSO-treated target cells and 15% for aphidicolin-treated target cells.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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