Induction of a pro-apoptotic ATM–NF- κ B pathway and its repression by ATR in response to replication stress

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The transcription factor NF- κ B has critical functions in biologic responses to genotoxic stimuli. Activation of NF- κ B in response to DNA double strand break (DSB) inducers can be mediated by ATM (ataxia telangiectasia mutated)-dependent phosphorylation of NEMO (NF- κ B essential modulator). Here, we show that the replication stress inducers hydroxyurea (HU) and aphidicolin also activate this ATM-dependent signalling pathway. We further show that ATR (ATM- and Rad3-related) interacts with NEMO but surprisingly does not cause NEMO phosphorylation. Consequently, ATR represses NF- κ B activation induced by replication stress. Reduction or increase of ATR expression by RNA interference or overexpression increased or reduced ATM–NEMO association and NF- κ B activation induced by HU. Apoptosis gene expression and chromatin immunoprecipitation analyses indicated that HU and the DSB inducer etoposide caused complex patterns of NF- κ B-dependent pro- and antiapoptotic gene expression with the overall outcome for the former being pro-apoptotic, whereas the latter antiapoptotic. Thus, replication stress and DSB inducers activate NF- κ B through a conserved pathway with opposite biologic outcomes, and ATR antagonizes ATM function at least in part by competing for NEMO association.

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Introduction

Genomic instability is a hallmark of cancer and ageing processes (Finkel *et al.*, 2007). One major threat to maintaining genome integrity comes from DNA-damaging agents either generated from endogenous cell metabolism (e.g. reactive oxygen species) or exogenously provided (e.g. chemicals or radiation). To cope with these threats, cells have evolved complex DNA damage responses, which include activation of cell-cycle checkpoints, DNA repair pathways and signal-trans-

duction pathways (Bartek and Lukas, 2007; Bartek *et al.*, 2007). DNA lesions can be detected by DNA damage sensors such as the Mre11–Rad50–NBS1 (MRN) complex and the Rad9–Rad1–Hus1 (9-1-1) complex, which in turn help to recruit and activate two major kinases capable of transducing DNA damage signals, ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR). In response to different forms of DNA damage, ATM and ATR phosphorylate a number of downstream effector proteins and kinases, such as the checkpoint kinases Chk1 and Chk2, which regulate cell-cycle arrest, DNA repair, apoptosis, gene transcription and senescence (Zhou and Elledge, 2000; McGowan and Russell, 2004; Bartek and Lukas, 2007). A recent study showed that two major transcription factors that are activated in certain normal murine tissues (e.g. lymph node) in response to whole body irradiation are pro-apoptotic tumour suppressor p53 and prosurvival NF- κ B (Rashi-Elkeles *et al.*, 2006).

The NF- κ B family of transcription factors consists of five members, RelA (p65), RelB, c-Rel, NF- κ B1/p50 and NF- κ B2/p52, which exist as either homo- or heterodimers (Ghosh and Karin, 2002; Perkins, 2007). The prototypical NF- κ B composed of a p50–p65 heterodimer is found in the cytoplasm of most cells in a latent form in association with its inhibitor I κ B. I κ B also belongs to a family of related proteins that include I κ B α , I κ B β , I κ B ϵ , I κ B γ , Bcl-3, NF- κ B1/p105 and NF- κ B2/p100. In response to various stimuli, the major I κ B family member I κ B α is phosphorylated by the I κ B kinase (IKK) complex, which leads to degradation of I κ B α by the ubiquitin–proteasome pathway (Karin and Ben-Neriah, 2000; Chen, 2005). Once I κ B α is degraded, NF- κ B undergoes rapid nuclear translocation and regulates expression of multiple target genes involved in a variety of physiological and pathological processes, including immune and inflammatory responses, proliferation and apoptosis (Karin and Ben-Neriah, 2000; Hayden and Ghosh, 2004).

The IKK complex contains two catalytic subunits, IKK α /IKK1 and IKK β /IKK2 and a regulatory subunit IKK γ /NEMO (NF- κ B essential modulator) (Ghosh and Karin, 2002; Perkins, 2007). This complex responds to a wide array of extracellular and intracellular stimuli and causes activation of NF- κ B through a so-called ‘canonical’ pathway. The canonical pathway activates p65- or c-Rel-containing homo- and heterodimers. There is also a ‘non-canonical’ pathway of NF- κ B activation (Hayden and Ghosh, 2004; Scheidereit, 2006). In this alternative pathway, IKK α , without the need for IKK β or NEMO, causes phosphorylation- and ubiquitination-dependent processing of NF- κ B2/p100 to p52 resulting in selective activation of p52/RelB heterodimers. The non-canonical pathway is activated by a smaller set of inducers and is important for development of secondary lymphoid organs, among others (Bonizzi and Karin, 2004).

Genotoxic agents used in cancer treatment, such as ionizing radiation (IR) and topoisomerase I and II inhibitors, can

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activate NF- κ B through an 'atypical' pathway that is initiated from the nucleus (Janssens and Tschoop, 2006; Scheidereit, 2006; Wu and Miyamoto, 2007). Several studies have shown that ATM is essential for NF- κ B activation by multiple genotoxic agents that induce DNA double strand breaks (DSBs), including IR, the topoisomerase I inhibitor camptothecin (CPT) and topoisomerase II inhibitors etoposide (VP16) and doxorubicin (Piret *et al*, 1999; Li *et al*, 2001; Huang *et al*, 2003; Wu *et al*, 2006). In the atypical pathway, NEMO without the IKK catalytic subunits enters the nucleus and associates with DSB-activated ATM (Wu *et al*, 2006). The nuclear localization of NEMO is regulated by its post-translational modification by SUMO-1 (small ubiquitin-like modifier 1) (Huang *et al*, 2003). This sumoylation step is under the control of PIDD (p53-induced protein with death domain) and RIP1 (receptor interacting protein 1) (Janssens *et al*, 2005). In addition, PIASy (protein inhibitor of activated STAT y) also has a critical function as a SUMO ligase that promotes NEMO sumoylation in response to DNA-damaging agents (Mabb *et al*, 2006). It is unclear how ATM-NEMO association is regulated in the nucleus; however, subsequent ATM-dependent phosphorylation of NEMO at serine 85 is critical for further NEMO modification by ubiquitin, NEMO nuclear export and activation of the canonical IKK complex (Wu *et al*, 2006). NF- κ B activation by DSB inducers then generally promotes cell survival by apparently turning on antiapoptotic genes (Wang *et al*, 1999; Baldwin, 2001; Dutta *et al*, 2006).

Besides DSBs, replication stress inducers, such as hydroxyurea (HU), are also used as anticancer drugs to arrest replication forks and cause death of malignant cells. Whereas ATR is believed to have the immediate and dominant function in cellular response to replication stress, ATM also participates in this type of stress with delayed kinetics (Abraham, 2001; Durocher and Jackson, 2001; Hurley and Bunz, 2007). Currently, it is unclear whether replication stress causes NF- κ B activation, and if so what mechanisms are involved and what the functional consequences are. Here, we provide several lines of genetic and biochemical evidence suggesting that replication stress inducers, HU and aphidicolin, activate NF- κ B in an ATM and NEMO-dependent manner similar to DSB inducers. Surprisingly, NF- κ B activation by replication stress promotes a genetic programme favouring cell death, rather than cell survival. Moreover, ATR prevents ATM-mediated NF- κ B activation response in part by competing for NEMO association without causing serine 85 phosphorylation. Our study highlights that ATM and ATR can have opposite functions with respect to NF- κ B signalling and biological functions.

Results

Replication-blocking agents activate NF- κ B in an ATM-dependent manner

To investigate whether replication stress inducers can activate NF- κ B, human HEK293 embryonic kidney cells, mouse 70Z/3 pre-B cells and human CEM T cells were treated with varying doses of HU and aphidicolin for different durations. As controls, these cells were also exposed to atypical inducers (VP16 and CPT) and canonical inducers (TNF α and LPS) of NF- κ B signalling. Whole-cell lysates were analysed by electrophoretic mobility shift assay (EMSA) using a radiolabelled κ B site from the immunoglobulin (Ig) κ intronic enhancer as a

probe. HU and aphidicolin induced NF- κ B activation to variable levels in these cell lines but the peak activation was generally lower than those induced by atypical and canonical inducers (Figure 1A, lanes 5 and 6). We also observed NF- κ B activation by replication stressors in mouse embryonic fibroblasts (MEFs) and human lymphoblast cells, again relatively weakly (see below). Supershift assays showed that the major NF- κ B complex was composed of p65-p50 heterodimers (Supplementary Figure 1). Not only did these agents cause generally lower NF- κ B activation but also did so with slower kinetics (Figure 1B and D). Accordingly, chromatin immunoprecipitation (ChIP) analysis indicated the delayed p65 recruitment to I κ B α κ B promoter after HU treatment compared with VP16 treatment condition (Figure 1C). We have previously provided evidence that VP16 and TNF α cause NF- κ B activation regardless of the cell-cycle phases (Wuerzberger-Davis *et al*, 2005). As HU and aphidicolin primarily disrupt the S phase of the cell cycle, the reduced NF- κ B activation may in part be due to the smaller target cell population. As described below, there is a repressive mechanism operating to keep this activation pathway limited as well.

DSB inducers activate both ATM and ATR robustly, but replication stress inducers rapidly activate ATR, whereas ATM to a lesser extent with a slower kinetics (McGowan and Russell, 2004; Stiff *et al*, 2006; Hurley and Bunz, 2007). NF- κ B activation by DSB inducers requires ATM, but it is unclear whether ATM or ATR is required for activation by replication stress inducers. To investigate the relationship between the kinetics of ATM and NF- κ B activation by replication stress inducers, we measured phosphorylation status of serine 1981 (pS1981) of ATM. Kinetics of pS1981-ATM generally correlated with NF- κ B activation by aphidicolin (Figure 1D, others not shown). Next, we used KU55933 that selectively inhibits the kinase activity of ATM but not ATR (Lau *et al*, 2005), to evaluate the function of ATM in replication stress-induced NF- κ B activation. NF- κ B activation by aphidicolin and HU was blocked by the ATM inhibitor (Figure 2B, others not shown). As a control for the inhibitor, we show that ATM activation as measured by pS1981-ATM antibody reactivity was effectively blocked by KU55933 (Figure 2A). The lack of significant ATM activation by HU in HEK293 cells (Figure 2A) correlated with the weak NF- κ B activation by this agent in this cell system (Figure 1A). The ATM inhibitor did not affect NF- κ B activation by TNF α or LPS (Figure 2B, others not shown).

To further evaluate the requirement of ATM in NF- κ B activation by replication stressors, we next evaluated ATM wild-type (ATM^{+/+}) and ATM knockout (ATM^{-/-}) MEF cells. LPS activated NF- κ B in both cell systems. In contrast, NF- κ B activation by CPT and HU was greatly diminished in ATM^{-/-} MEFs compared with that in ATM^{+/+} MEFs (Figure 2C). Furthermore, both VP16- and HU-induced NF- κ B activation was absent in an AT patient-derived human lymphoblast cell line AT59 compared with the ATM wild-type lymphoblast cell line L-40 where activation was evident (Figure 2D). Finally, NF- κ B activation induced by VP16 and aphidicolin was significantly inhibited by ATM inhibitor KU55933 in human primary peripheral blood CD4⁺ T cells (Figure 2E). All together, these results indicate that ATM is generally required for NF- κ B activation by replication stress inducers.

Figure 1 Activation of NF- κ B by replication stress inducers. (A) HEK293, CEM and 70Z/3 cells were treated with TNF α (10 ng/ml, 30 min), LPS (10 μ g/ml, 30 min), VP16 (10 μ M, 2 h), CPT (10 μ M, 2 h), HU (2 mM, 3 h) or aphidicolin (Aph; 10 μ g/ml, 3 h), and whole-cell lysates were analysed by EMSA using Ig κ - κ B and Oct-1 probes. (B) 70Z/3 cells were exposed to VP16 (10 μ M) or HU (2 mM) for indicated times. Whole-cell lysates were analysed by EMSA for NF- κ B activation. Relative intensities of NF- κ B DNA binding were acquired by phosphorimage analysis, normalized to untreated samples and fold induction was plotted. (C) 70Z/3 cells were treated with VP16 or HU as shown. Recruitment of p65 on κ B promoter of I κ B α was analysed with ChIP and semiquantitative PCR. (D) HEK293 cells were treated with VP16 (10 μ M) or aphidicolin (Aph; 10 μ g/ml) and analysed as in (B). The whole-cell lysates were also subjected to western blotting analysis using anti-pS1981-ATM and anti-ATM antibodies.

IKK activation and I κ B α degradation are required for replication stress-induced NF- κ B activation

IKK kinase activity is critical for NF- κ B activation by DSB inducers (Li and Karin, 1998; Wu *et al.*, 2006). To determine if replication stress also leads to IKK activation, 70Z/3 cells were treated with HU and aphidicolin for 150 min, and processed for an IKK immunocomplex kinase assay. The cells were also treated with VP16 for 90 min as a positive control. Both HU and aphidicolin induced IKK kinase activity as measured by phosphorylation of wild type (Figure 3A, lanes 3–5) but not S32/36A mutant I κ B α protein (Figure 3A, lane 6). IKK activation was also induced by HU in wild-type MEF but not in ATM^{-/-} MEF cells (Figure 3B), demonstrating that ATM functions upstream of IKK to cause NF- κ B activation similar to DSB-inducing agents (Li and Karin, 1998; Huang *et al.*, 2003; Wu *et al.*, 2006). Consistent with the activation of IKK complex, NF- κ B activation by HU and aphidicolin was blocked by the presence of super-repressor S32/36A-I κ B α (Figure 3C). Finally, both HU and aphidicolin caused degradation of I κ B α , which was prevented by the proteasome inhibitor MG132 (Figure 3C and D). Inhibition of I κ B α degradation abrogated NF- κ B activation by HU and aphidicolin. Similar to DSB inducers, NF- κ B activation by replication stressors was not inhibited by cycloheximide treatment, indicating that *de novo* protein synthesis was not a requirement for this activation pathway (Figure 3D). These results demonstrate that replication stressors activate NF- κ B through IKK-dependent and proteasome-mediated I κ B α degradation, similarly to DSB inducers.

NEMO sumoylation and phosphorylation are also required for NF- κ B activation by replication stress inducers

We previously showed that specific modifications of NEMO are required for NF- κ B activation by DSB inducers (Huang *et al.*, 2003; Wu *et al.*, 2006). As our study thus far suggested that a similar pathway is induced by HU and aphidicolin to cause NF- κ B activation, we next evaluated the requirement of NEMO, the regulatory subunit of the IKK complex, and its known post-translational modifications. 1.3E2 pre-B cells deficient for NEMO did not activate NF- κ B in response to HU and aphidicolin (Figure 4A), whereas stable expression of Myc-tagged NEMO restored activation. NEMO knockdown by siRNA in HEK293 cells also resulted in the inhibition of NF- κ B activation by aphidicolin (Figure 4B). We next employed 1.3E2 cells stably expressing different NEMO mutants to evaluate whether specific domains and amino-acid residues that are required for NF- κ B activation by DSB inducers (Huang *et al.*, 2003; Wu *et al.*, 2006) are also required for replication stress inducers. The ATM site S85 (and the critical Q86 residue), the putative sumoylation sites K277/K309, and C-terminal zinc-finger domain of NEMO were all found to be essential for NF- κ B activation by HU and aphidicolin (Figure 4C–E). The results from these analyses are summarized in Figure 4F, which highlights that all the NEMO sequences critical for NF- κ B activation by DSB inducers are also essential for activation by HU and aphidicolin.

To test whether NEMO sumoylation and phosphorylation are induced by replication stressors, HEK293 cells stably

Figure 2 Replication stress-induced NF- κ B activation is ATM dependent. (A) HEK293 cells were treated with VP16 (10 μ M, 2 h), aphidicolin (Aph; 10 μ g/ml, 3 h) or HU (2 mM, 3 h) in the presence or absence of KU55933 (KU; 10 μ M). Whole-cell lysates were subjected to western blot analysis using anti-pS1981-ATM and anti-ATM antibodies. OT: untreated. (B) 70Z/3 cells were treated with LPS (L), VP16 (V) or HU (H) as in Figure 1A in the presence of KU55933 or DMSO, and whole-cell lysates were analysed by EMSA. (C) ATM^{-/-} and ATM^{+/+} MEFs were treated with LPS (L), CPT (C) or HU (H) as above, and whole-cell lysates were analysed by EMSA and western blotting using anti-ATM and anti-ATR antibodies. (D) A similar analysis as in (C) was carried out using AT59 (ATM deficient) and L-40 (ATM WT) human lymphoblast cells. (E) Human primary CD4⁺ PBL-T cells were treated as in (A), whole-cell lysates were analysed with EMSA.

expressing Myc-NEMO were exposed to VP16 and aphidicolin for 0, 60 and 90 min and processed for sumoylation assay (Huang *et al.*, 2003). Aphidicolin induced NEMO sumoylation similarly to VP16 (Figure 5A, lanes 3 and 6). Repeated analyses along with quantitation indicated that the kinetics and the magnitude of NEMO sumoylation were similar between aphidicolin and VP16 treated samples (Figure 5A, graph). Furthermore, we also found that S85 of NEMO was phosphorylated after aphidicolin treatment (Figure 5B, lanes 11 and 12) with a delayed kinetics, mirroring pS1981-ATM detection and the phosphorylation of ATM substrate CREB at S121 (Dodson and Tibbetts, 2006). In contrast, phosphorylation of Chk1 at S317, a marker of ATR activation, peaked within 15 min of aphidicolin treatment (lane 8). Apparently, rapid activation of ATR did not lead to NEMO phosphorylation, similar to the situation with CREB (Dodson and Tibbetts, 2006).

N-terminal IKK-interacting region of NEMO associates with the ATM FAT domain

The domains of ATM and NEMO that are involved in their mutual interaction are presently unclear. To delineate the region of ATM required for NEMO binding, three major ATM domains, the ATM/ATR homologous domain (HD), the FAT domain and the PI3K-like kinase domain (PK) (depicted in Figure 6A), were translated *in vitro* in the presence of ³⁵S-methionine and subjected to a GST pull-down assay as described previously (Park *et al.*, 2005) using GST-NEMO. We found that only the FAT domain was able to specifically associate with NEMO (Figure 6A). To determine the region

of NEMO that is required for ATM association, HEK293 cells were transiently transfected with a series of NEMO deletion mutants (depicted in Figure 6C) along with an ATM-FAT domain expression construct. Through co-immunoprecipitation, a minimal region of NEMO required for ATM interaction mapped to the N-terminal 1–106 amino-acid domain (Figure 6B). Intriguingly, this binding region overlaps with that required for NEMO association with IKK β (60–120) and PIASy (1–126) (Mabb *et al.*, 2006; Marienfeld *et al.*, 2006) (Figure 6C). Accordingly as in the case with PIASy, NEMO-ATM and NEMO-IKK β interactions were competitive under overexpression conditions, where both IKK β and NEMO distribute to nucleus and cytoplasm. Decreased NEMO-ATM and increased NEMO-IKK β interaction was observed as IKK β expression was increased (Figure 6D). These results further support the notion that an overlapping N-terminal NEMO sequence is used for interaction with ATM and IKK β .

ATR can also associate with NEMO through the FAT domain but does not cause S85 phosphorylation of NEMO and prevents ATM-mediated NF- κ B activation

Replication stress rapidly induces peak ATR activation, yet we did not observe rapid pS85 NEMO immunoreactivity (Figure 5B). We therefore wanted to determine what role ATR might have in NF- κ B signalling in response to replication stress. When its expression was depleted by ATR-specific siRNAs in HEK293 cells, NF- κ B activation by HU was significantly increased (Figure 7A and Supplementary Figure 2A). Normally, aphidicolin-induced NF- κ B activation is only barely detectable by EMSA analysis, but with ATR knock-

Figure 3 Replication stress inducers cause IKK activation and promote proteasome-dependent degradation of I κ B α . (A) 70Z/3 cells were treated with VP16 (V; 10 μ M, 2 h), aphidicolin (A; 10 μ g/ml, 3 h), HU (H; 2 mM, 3 h) or left untreated (–). Cell lysates were subjected to immunoprecipitation with anti-NEMO antibody and IKK kinase assay was performed as described in Materials and methods using GST–I κ B α -WT or -S32/36A mutant protein as substrates. IKK kinase activity was measured with Phosphorimager and presented as mean $\pm$ s.d. for three independent experiments. (B) ATM^{+/+} and ATM^{-/-} MEFs were treated with LPS, HU or left untreated as in (A). Whole-cell lysates were analysed as in (A). (C) 70Z/3 cells stably expressing I κ B α -WT or -S32/36A mutant were treated as in Figure 1A. Whole-cell lysates were subjected to EMSA and western blotting with anti-I κ B α . Top panels were assembled from the same gel of the same exposures with the break in the lanes denoted by a black line. *Exo: exogenous I κ B α , **Endo: endogenous I κ B α . (D) 70Z/3 cells were treated as in (C) in the presence of cycloheximide (Cx; 10 μ M) or MG132 (10 μ M). Whole-cell lysates were analysed as in (C).

down it became evident (Figure 7A). These results suggested the possibility that ATR might function to inhibit NF- κ B activation in response to replication stress. Moreover, anti-NEMO antibody, but not control IgG, was able to co-immunoprecipitate ATR in HEK293 cells exposed to aphidicolin or HU (Figure 7B). An ATR-binding partner ATRIP was also found in the NEMO immunocomplex. Consistent with the weaker NF- κ B activation by HU, NEMO–ATR association was also lower in HEK293 cells. As FAT domains of ATM and ATR are conserved (Abraham, 2001; Park *et al.*, 2005) and ATM–FAT is involved in NEMO interaction (Figure 6A), we tested if the ATR–FAT domain also bound to NEMO. ATR–FAT could associate with GST–NEMO (Supplementary Figure 2B). When ATM–FAT and ATR–FAT domains were added together in the GST pull-down assay, GST–NEMO reproducibly associated with ATM–FAT $\sim$ 3-fold more efficiently than ATR–FAT (Supplementary Figure 2B), indicating that ATM has a higher affinity for NEMO than ATR does. Finally, hyperactivation of replication stress-induced NF- κ B activation in response to ATR knockdown was accompanied by increased NEMO–ATR interaction (Figure 7C, others not shown). These results indicated that both ATM and ATR associates with NEMO under replication stress conditions and reduction of ATR expression leads to increased ATM–NEMO association. They also suggested that early activation of ATR by replication stress allows its association with NEMO, albeit with a weaker affinity, to reduce the available NEMO for binding with ATM that is activated at later times. This

competition for NEMO leads to ATR-mediated repression of NF- κ B response.

The above ATR competition model predicted several outcomes. First, if the expression of ATR was increased progressively, interaction between ATM and NEMO should correspondingly decrease. Second, under the above experimental setting, we should also observe progressive decrease in NF- κ B activation by replication stress inducers. When Flag–ATR was introduced transiently into HEK293 cells stably expressing Myc–NEMO and treated with aphidicolin, NEMO–ATR association increased in accordance with the decrease of NEMO–ATR association (Figure 7D). EMSA analysis demonstrated that this increase of ATR–NEMO binding decreased NF- κ B activation (Figure 7E). These results are consistent with the ATR competing with ATM for NEMO binding and reveal at least one mechanism that explains why NF- κ B activation by replication stress inducers is generally weak and delayed compared with DSB inducers that can activate ATM rapidly and robustly.

NF- κ B activation by replication stress promotes cell death through shifting the balance of apoptosis-related gene expression

NF- κ B activation by various DSB inducers is linked to protection of cells from apoptosis by activating antiapoptotic genes (Scheidereit, 2006; Wu and Miyamoto, 2007). We have also demonstrated that IR-induced NF- κ B activation is associated with increased cell survival as measured by clonogenic

Figure 4 Mutational analysis of critical NEMO residues required for NF- κ B activation by replication stress. (A) NEMO-deficient 1.3E2 cells or those stably reconstituted with NEMO-WT were treated with LPS (L; 30 min), VP16 (V; 2 h), HU (H; 3 h), aphidicolin (A, 3 h) or left untreated (–). Whole-cell lysates were analysed by EMSA and western blotting with anti-NEMO and anti-tubulin antibodies. (B) HEK293 cells were mock transfected or transfected with NEMO siRNAs, and analysed as in (A). Relative intensities of radioactive signals were obtained by Phosphorimager analysis and shown as fold induction. T: TNF α . (C) NEMO-WT and -S85A cells were treated as in Figure 1A and analysed by EMSA. (D) A similar analysis as in (C) was carried out using NEMO-Q86A and -Q86N cells. All the EMSA panels in (B–D) were assembled from the same corresponding gels of the same exposures with the breaks in the lanes denoted by black lines. (E) NEMO-K277/309A cells were treated as in (C) and analysed by EMSA. (F) A summary of NEMO residues required for NF- κ B signalling induced by different agents.

survival assay (Wu *et al.*, 2006). To examine the role of NF- κ B activation in response to replication stress, we next performed clonogenic survival assay using 1.3E2 cells reconstituted with either NEMO-WT or NEMO-S85A mutant. NF- κ B is activated by both VP16 and HU in NEMO-WT but not in NEMO-S85A cells (Figure 4C). In sharp contrast to VP16 and IR treatment (Wu *et al.*, 2006), where cell survival is higher in NEMO-WT cells (NF- κ B activation competent) than in NEMO-S85A cells (NF- κ B activation incompetent), HU surprisingly caused more death in NEMO-WT cells than in NEMO-S85A cells (Figure 8A). We also observed more cell death in HEK293 cells in response to HU treatment than those incapable of activating NF- κ B due to stable expression of the super-repressor I κ B α mutant (Supplementary Figure 3). These results demonstrated that NF- κ B activation in response to replication stress promoted rather than inhibited cell death.

To determine the basis for NF- κ B-dependent induction of cell death following HU treatment, we next employed a RT-PCR array system focused on an apoptosis gene cluster to screen for NF- κ B target genes whose expression is altered by HU versus VP16 treatment. This analysis suggested that both HU and VP16 induced a host of both pro-apoptotic (Apaf1, certain caspases, Fas and Fas-L), antiapoptotic (Bcl2 family members), and B-cell activation and differentiation-related genes (LT β R and CD40), among others to variable levels

(Supplementary Table 1). To determine NF- κ B dependence of pro- and antiapoptotic gene induction, we chose four well-established NF- κ B-regulated apoptosis genes (Fas, Fas-L, Bcl2l1/Bcl-X_L and Bcl2) to further probe the mechanism of NF- κ B-dependent responses after HU and VP16 treatment. Fas was robustly upregulated by both HU and VP16 treatments, whereas Bcl-X_L was upregulated only by VP16 exposure but not by HU (Figure 8B). Bcl2 was not induced by either stimulus. The comparison of the induction of these genes between WT- and S85A-NEMO-expressing cells demonstrated that these genes were induced in an NF- κ B-dependent manner (Supplementary Figure 4).

Previous studies have shown that expression of an anti-apoptotic gene Bcl-X_L is repressed by p65 through ATR-CHK1-mediated phosphorylation in response to p14^{ARF} overexpression or in response to UV treatment, thereby promoting NF- κ B-dependent cell death (Campbell *et al.*, 2004; Rocha *et al.*, 2005). In response to HU, we failed to observe decreased expression of Bcl-2 and Bcl-X_L genes as measured by quantitative real-time PCR in either NEMO-WT or NEMO-S85A cells, but Bcl-X_L gene was not induced by HU treatment (Figure 8B and Supplementary Figure 4). Thus, HU treatment situation differs from those previously described for UV and p14^{ARF} above. To further delineate the mechanism of the differential NF- κ B regulation of Fas and Bcl-X_L gene transcription under a replication stress condition, we next employed

Figure 5 NEMO is sumoylated and phosphorylated in response to replication stress inducers. **(A)** HEK293 cells stably expressing Myc-NEMO were exposed to VP16 (10 μ M) or aphidicolin (10 μ g/ml) for indicated times. Cell extracts were boiled in lysis buffer containing 1% SDS and immunoprecipitated with anti-Myc antibody. Immunoprecipitates were analysed by western blotting using anti-SUMO-1 antibody. Data from three independent experiments were analysed with ImageJ, and fold induction of sumo band intensity were plotted as mean $\pm$ s.d. **(B)** HEK293 cells stably expressing Myc-NEMO were exposed to VP16 (10 μ M) or aphidicolin (10 μ g/ml) for indicated times as shown. Whole-cell lysates were subjected to western blotting analysis using antibodies as indicated.

ChIP assays. We found that p65, the major NF- κ B component activated by HU and VP16 (Supplementary Figure 1), was recruited to the promoter regions containing κ B sites in both of these genes in response to both VP16 and HU treatments (Figure 8C and D). On the κ B promoter region of Fas, both VP16 and HU treatment induced CBP binding, which correlated with the induction of Fas by both treatments (Figure 8C). Surprisingly, only VP16, but not HU, enriched the co-activator CBP on the κ B promoter region of the antiapoptotic gene Bcl-X_L. Instead, HU treatment enhanced the binding of a transcriptionally repressive HDAC1 on the Bcl-X_L promoter region, correlating with the lack of induction of this key antiapoptotic gene by HU treatment (compare Figure 8B and D). Our results demonstrate a surprising finding that both VP16 and HU induced complex transcriptional programmes that include both pro- and antiapoptotic genes. Remarkably, NF- κ B activation by VP16 that is overall pro-survival robustly induced pro-apoptotic genes, such as

Fas and FasL, similarly to pro-apoptotic NF- κ B activation induced by HU. However, there is differential regulation of critical NF- κ B-mediated antiapoptotic genes, such as that highlighted by Bcl-X_L that is induced by VP16 but not by HU due to differential recruitment of transcriptional co-regulators. We propose that the overall survival function of NF- κ B in response to genotoxic stimuli, such as replication stress and DSB inducers, depends on the balance of the induction levels of pro- and antiapoptotic genes.

Discussion

Our understanding of functions and mechanisms of NF- κ B activation by DSB-inducing agents has substantially increased over the last two decades (Wang *et al.*, 1996; Li and Karin, 1998; Criswell *et al.*, 2003; Janssens and Tschopp, 2006; Wu and Miyamoto, 2007). In contrast, those pertaining to NF- κ B signalling induced by replication stress inducers remain relatively unexplored. In the present study, we provided several lines of evidence indicating that the replication stressors HU and aphidicolin induce NF- κ B activation through a conserved pathway that is also employed by DSB-inducing agents. First, ATM is the DNA damage responsive kinase required for NF- κ B signalling in response to replication stress. Second, replication stressors induce IKK kinase activity leading to proteasome-dependent degradation of I κ B α . Third, NEMO sumoylation and S85 phosphorylation are induced by replication stress agents. And finally, NEMO mutants that selectively prevent DSB-induced NF- κ B activation, but not cytokine- or LPS-induced activation, also abrogate HU/aphidicolin-induced NF- κ B activation. Taken together, our results are consistent with the idea that both replication stress and DSB inducers activate NF- κ B through a conserved ATM-mediated signalling pathway.

One surprising result from the current study is that ATM, rather than ATR, is the essential DNA damage signal transducer for NF- κ B activation in response to replication stress. ATR is generally thought to be the main replication stress-responsive kinase that mediates cellular DNA damage checkpoint responses (Abraham, 2001; Durocher and Jackson, 2001; McGowan and Russell, 2004). Accordingly, we found evidence for rapid and robust ATR activation as measured by pS317-Chk1 that reached maximally as early as 15 min after aphidicolin treatment (Figure 5B). However, detectable pS85-NEMO did not appear until 2 h after the treatment, correlating with late activation of ATM. Recent studies have also demonstrated that ATM can be activated in response to treatment with replication stressors such as HU and aphidicolin (Dodson and Tibbetts, 2006; Stiff *et al.*, 2006; Ozeri-Galai *et al.*, 2007). The mechanism of ATM activation in response to replication stress remains controversial. ATM activation by UV and HU has been shown to require ATR kinase activity, indicating that ATR is upstream of ATM (Stiff *et al.*, 2006). It has also been shown that ATM is activated by aphidicolin in an ATR-independent manner in HeLa cells (Ozeri-Galai *et al.*, 2007). Finally, increased phosphorylation of S1981 of ATM has been shown in ATR-seckel mutant (ATR deficient) cells compared with that in wild-type cells (Hurley and Bunz, 2007). We found that knock down of ATR did not block ATM-dependent NF- κ B activation (Figure 7), indicating that ATR is not functioning upstream of ATM under our experimental conditions. The lack of ATR involvement in the NF- κ B

Figure 6 NEMO N-terminal region associates with the FAT domain of ATM. (A) ATM HD, FAT and PK domains were *in vitro* translated in the presence of ^{35} S-methionine. Bacterially expressed GST and GST-NEMO proteins were used for *in vitro* binding assay as described in Material and Methods, analysed by SDS-PAGE and exposed to Phosphorimager screen. Lanes 1, 4 and 7 represent 5% input proteins. GST-tagged proteins were stained with Coomassie blue. (B) Myc-tagged NEMO truncation mutants were transiently transfected along with HA-ATM-FAT in HEK293 cells. Whole-cell lysates were analysed by co-IP using anti-HA antibody and western blotting with anti-Myc and anti-HA antibodies. Arrow indicates HA-ATM; *positions in input gel. Black dots indicate heavy and light chains. (C) A diagram depicting an ATM-binding region of NEMO at the N-terminal region. This region overlaps with NEMO-binding regions for IKK β and PIASy. (D) Myc-NEMO stable HEK293 cells were transfected with HA-ATM along with increasing amounts of Flag-IKK β . Co-IP experiment was carried out as in (A) and analysed with antibodies as shown.

signalling was not due to its lack of interaction with NEMO, as ATR-NEMO could be detected *in vivo* and its FAT domain interacted with NEMO, similar to ATM *in vitro* (Figure 7 and Supplementary Figure 2). Instead, our data demonstrated that ATR antagonizes NF- κ B activation by replication stress. Thus, knock down of ATR not only failed to block but rather enhanced ATM-dependent NF- κ B activation.

On the basis of our findings, we propose the following model. At an early stage after replication stress induction, ATR is robustly activated with little amount of activated ATM in the nucleus. This situation promotes preferential association of ATR and NEMO that is now accumulating in the nucleus in association with its sumoylation. As ATM activation is increased, ATM associates with NEMO. Our *in vitro* data indicated that ATM has higher affinity for NEMO than ATR (Supplementary Figure 2); thus, ATM might even compete with ATR for NEMO association in the nucleus as its

activity increases. Finally, ATM induces S85 phosphorylation of NEMO to ultimately cause NF- κ B signalling. Thus, a decrease or increase of ATR level can correspondingly increase or decrease ATM-dependent NF- κ B activation. Moreover, this competition between ATM and ATR is not limited only to replication stress conditions. In response to ATR knockdown, we have also observed increased NF- κ B activation by IR and VP16. We have also found that different cancer cell lines express variable levels of ATM and ATR and the differences in these expression levels can be striking (S Wuerzberger-Davis and S Miyamoto, unpublished observations). The competition of NEMO association between ATR and ATM is probably not the only mechanism of ATR-mediated inhibition of NF- κ B signalling. For example, the depletion of ATR may have secondary effects of inducing DNA lesions and possibly augment NF- κ B activation due to heightened ATM activation under certain cellular settings.

Figure 7 ATR inhibits replication stress-induced NF- κ B activation through competing NEMO association with ATM. (A) HEK293 cells were mock transfected or transfected with siRNAs targeting ATR. EMSA and western blotting analysis were carried out as in Figure 4B using antibodies against ATM and ATR. EMSA data from triplicate experiments were graphed as mean $\pm$ s.d. (B) HEK293 cells were treated with HU (2 mM), aphidicolin (Aph; 10 μ g/ml) for 150 min or left untreated. Whole-cell lysates were precipitated with α -NEMO (N) antibody or control IgG (G) and followed by western blot analysis with antibodies as indicated. (C) HEK293 cells stably expressing Myc-NEMO were transfected with control or ATR siRNAs. After 48 h, cells were treated with aphidicolin (Aph; 10 μ g/ml) for 150 min. Total cell extracts were immunoprecipitated with anti-Myc antibody. Precipitates were examined by western blotting using antibodies against ATM, ATR and Myc. (D) Myc-NEMO stable HEK293 cells were transfected with increasing amounts of Flag-ATR. Cells were treated with aphidicolin (10 μ g/ml) for 150 min, and Co-IP experiment was carried out as in Figure 6D and analysed with antibodies as shown. (E) HEK293 cells were transfected and treated as in (D). Whole-cell lysates were analysed with EMSA and western blot as indicated.

Figure 8 NF- κ B activation in response to replication stressors or DSB inducers differentially regulates gene expression. (A) NEMO-WT and NEMO-S85A cells were treated with HU (3 mM) or VP16 (VP; 10 μ M) for indicated times. Clonogenic survival assays were carried out as described in Materials and methods. Cell survival fractions were plotted as average $\pm$ s.d. from triplicate experiments. (B) 70Z/3 cells were treated with HU (3 mM), VP16 (10 μ M) for 5 h or left untreated. Gene expression of indicated NF- κ B target genes were analysed with quantitative RT-PCR. (C) 70Z/3 cells were treated as in (B). ChIP analysis was carried out using antibodies against p65, CBP or HDAC1. Recruitment of respective transcription factors on κ B promoter of Fas gene was analysed with quantitative PCR. (D) Similar ChIP analysis of transcription factor recruitment on κ B promoter of Bcl-X_L gene was performed as in (C).

Nevertheless, our findings highlight the need for further evaluations of relative expression of ATM and ATR in cancer cell models with respect to NF- κ B signalling in response to genotoxic stimuli. Such analyses may help to understand why certain cell systems are more susceptible to NF- κ B activation by genotoxic stimuli, whereas others are not.

Although we found that both replication stress and DSB inducers share a common NF- κ B signalling pathway, the biologic outcomes of NF- κ B activation were surprisingly opposite in our current study. Although VP16 has been shown to induce apoptosis of Jurkat T cells through NF- κ B-mediated upregulation of FasL gene (Kasibhatla *et al*, 1998), many previous studies demonstrated a generally pro-survival effect of NF- κ B activation induced by IR and other DSB-inducing chemotherapeutic drugs by primarily inducing anti-apoptotic genes (Wang *et al*, 1996; Baldwin, 2001; Wu and Miyamoto, 2007). We also found that NF- κ B activation induced by IR promoted cell survival in mouse 1.3E2 pre-B cells (Wu *et al*, 2006). Surprisingly, in the case of HU treatment, NEMO-S85A cells that are incapable of activating NF- κ B with this stimulus survived better than NEMO-WT cells (Figure 8A). In the same cells, VP16 induced more cell death than in NEMO-WT cells. These results demonstrated that NF- κ B activation by HU and VP16 is associated with opposite survival outcomes in these cells. Through an unbiased screen of apoptosis-related gene expression, we found different gene expression profiles in the same cell system treated with HU or VP16 (Supplementary Table I). Among these, some pro-apoptotic genes such as Fas and FasL were induced by both HU and VP16 treatments, and the induction of these genes was clearly NF- κ B dependent (Supplementary Figure 4). However, the antiapoptotic gene Bcl-X_L was only induced by NF- κ B in response to VP16 but not by HU (Figure 8). This difference in Bcl-X_L regulation was correlated with the recruitment of the co-activator CBP to the promoter region by VP16 but not by HU. Instead, HU recruited a transcriptional repressor, HDAC1 to this promoter. This is analogous to previous studies that demonstrated that NF- κ B (p65) could function as a transcriptional repressor of Bcl-X_L either through ATR-Chk1-mediated phosphorylation of p65 or an unknown mechanism promoting NF- κ B association with HDAC in response to treatments with UV, daunorubicin or cisplatin and p14^{ARF} overexpression in U2OS osteosarcoma cells (Campbell *et al*, 2004, 2006; Rocha *et al*, 2005). The major distinction between these earlier studies and our present one is that NF- κ B activated by both VP16 and HU cause induction of pro-apoptotic genes as well. Thus, p65 did not function exclusively as a transcriptional repressor in response to HU treatment or an activator of only antiapoptotic genes in response to VP16 treatment. We also found that p21^{clp1/waf1} (Wuerzberger-Davis *et al*, 2005) and Bcl-X_L (data not shown) were upregulated in predominantly in S-G₂ phase and all cell-cycle phases, respectively, after VP16 exposures. Thus, this excludes the scenario where the lack of induction of Bcl-X_L by replication stress is due to the incapability of NF- κ B to induce this gene in the S phase. Instead, the final outcome of NF- κ B activation by DNA-damaging agents on cell survival is probably determined by the balance of a large pool of antiapoptotic and pro-apoptotic genes expression, which are either activated or repressed in response to specific genotoxic stimulus in a gene-selective manner in different

cell-cycle phases. Accordingly, a recent study highlighted the complexity of NF- κ B-dependent gene expression in different cell-cycle phases (Barre and Perkins, 2007).

Our current study combined with previous ones (Campbell *et al*, 2004; Rocha *et al*, 2005; Perkins and Gilmore, 2006; Wu and Miyamoto, 2007) demonstrate the complexity of NF- κ B functions in response to genotoxic stimuli. NF- κ B inhibition might be beneficial for increasing the efficacy of DSB-inducing anticancer agents. In contrast, our data suggest that such treatment might be counterproductive when replication stress inducers are used to treat cancer patients. It has been demonstrated that DNA damage responses take place at an early precancerous stage of human tumorigenesis (DiTullio *et al*, 2002; Bartkova *et al*, 2005; Gorgoulis *et al*, 2005). Oncogene activation and inappropriate proliferation have also been shown to cause DNA damage responses (Halazonetis *et al*, 2008). As part of these responses, NF- κ B could be activated early in cancer development. Constitutive activation of NF- κ B is a frequently observed phenomenon in human malignancies (see www.nf-kb.org) and inappropriate DNA damage responses might be in part responsible for this. Depending on the cancer cell context, NF- κ B activation in precancerous cells may have a pro-survival function to promote malignant conversions or pro-apoptotic role to enhance cell death to eliminate them. Thus, currently pursued anti-NF- κ B pathway drugs may also have either negative or positive impacts on secondary cancer development due to DNA damage responses. Further understanding of NF- κ B's role in genotoxic signalling may help to tailor beneficial outcomes while minimizing unwanted side effects.

Materials and methods

In vitro binding assay

In vitro binding assay of NEMO and ATM/ATR was carried out as described (Park *et al*, 2005). Briefly, ATM/ATR HD, FAT domain and PK domain PK were *in vitro* translated in the presence of 20 μ Ci of ³⁵S-methionine using TnT-coupled reticulocyte lysate system (Promega). GST and GST-NEMO were expressed in BL-21 cells and purified with glutathione beads. *In vitro* binding was carried out in binding buffer (PBS containing 0.5% TX-100, 0.5 mM EDTA and 0.5 mM PMSF) for 4 h at 4°C. Binding complex was precipitated with glutathione beads, and resolved on 12.5% SDS-PAGE. Gel was dried and exposed to Phosphorimager screen or film.

ChIP assay

ChIP assay was carried out using ChIP assay kit (Upstate) as described previously (Chang *et al*, 2006). Quantitative real-time PCR data are presented by setting the control IgG-precipitated samples as unity. The average and s.d. values were calculated and plotted by the Microsoft Excel program. Antibodies used for ChIP are from Santa Cruz: anti-p65 (sc-372), anti-CBP (sc-369) and anti-HDAC1 (sc-6298). Primers used for quantitative RCR are listed in Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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