THE ROLE OF INTERFERON REGULATORY FACTORS IN REGULATING THE EXPRESSION OF NKG2D LIGANDS

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Table of Contents

List of Figures

List of Abbreviations

Ara-C: Cytarabine Arabinoside

ATM: Ataxia Telangiectasia Mutated

ATR: Ataxia Telangiectasia and Rad3-related protein

CCL5: Chemokine (C-C motif) ligand 5

DDR: DNA Damage Response

DMSO: Dimethyl Sulfoxide

DRAF-1: Double-stranded RNA-activated Transcription Factor

IFN: Interferon

IKKε: IκB Kinase epsilon

IP-10 (CXCL10): Interferon gamma-induced Protein (C-X-C motif Chemokine 10)

IRF: Interferon Regulatory Factor

ISG15: Interferon-Stimulated Gene 15

ISRE: Interferon-Stimulated Response Element

MDM2: Murine Double Minute 2

MHC: Major Histocompatibility Complex

MICA: MHC Class I polypeptide-related sequence A

MICB: MHC Class I polypeptide-related sequence B

NF-κB: Nuclear Factor of kappa light polypeptide gene enhancer in B-cells

NKG2D: NK group 2, member D)

RAET1: Retinoic Acid Early Transcript 1

SIKE1: Suppressor of IKKε 1

TBK1: TANK-binding kinase 1

ULBP: UL16 Binding Protein

Summary

The DNA damage response (DDR) is a cellular response to genotoxic stress that triggers cell cycle arrest and DNA repair mechanisms. It has been previously shown that NKG2D ligands are upregulated by the DDR in a p53-independent manner. We provide evidence in this study that the upregulation of NKG2D ligand expression in response to DNA damage depends on the serine/threonine kinase TBK1 and its phosphorylation target IRF3. The activation of IRF3 in response to DNA damage was evidenced by its phosphorylation and nuclear translocation. TBK1 is upstream of IRF3 and similarly, its phosphorylation was observed during DNA damage. The pharmacological inhibition or knockdowns of either IRF3 or TBK1 reduced the DNA damage-mediated induction of NKG2D ligands. The overexpression of Sike1, an inhibitor of TBK1, abrogated the DNA damage-mediated expression of NKG2D ligands. IRF3 and TBK1 are also required for the maintenance of constitutive NKG2D ligand expression on tumour cell lines. The DNA damage sensor ATR was found to be implicated in IRF3 and TBK1 activation as inhibition of ATR kinase activity reduced the DNA damage-induced phosphorylation of IRF3 and TBK1. It remains to be elucidated if the ATR can directly phosphorylate TBK1, but the observation that phospho-ATM co-localized with Sike1 during DNA damage hinted that both ATM and ATR may be required for activation of the pathway and that the link from the DNA damage sensors to TBK1 is complex and indirect. These findings allow us to propose that genotoxic stress results in the activation of the TBK1/IRF3 pathway.

CHAPTER 1: INTRODUCTION

1.1 The role of IRFs in type 1 interferon induction

The innate immune system is coordinated by an intricate network of receptors, transcription factors, gene mediators and effectors. The role of type 1 interferons (IFN-α and IFN-β) in antiviral responses has been well documented (Honda and Taniguchi, 2006). The binding of type 1 IFN to surface IFN receptors initiates downstream signaling which leads to the induction of more than 300 IFN-stimulated genes (ISGs) (Der et al, 1998). Many of these ISGs modulate signaling pathways, pattern-recognition receptors or transcription factors to form positive feedback loops that result in the production of more interferons. Other IFN-inducible genes have direct antiviral activity such as inducing apoptosis of infected host cells and viral RNA degradation. Key to promoting type 1 IFN transcription are certain members of the family of transcription factors called interferon-regulatory factors (IRFs) (Honda and Taniguchi, 2006).

The role of IRFs first came into light during the study of interferon-β (IFN-β) induction by viruses, when it was first discovered that the expression of an unknown nuclear factor was induced by the Newcastle disease virus (NDV) in mouse fibroblast cells (Miyamoto et al, 1988). Using DNAse1 footprinting analysis, they found that this nuclear factor, which they termed IRF1, could bind to a regulatory region on IFN-β which correlated to its efficient expression (Miyamoto et al, 1988). This result highlighted the existence of an IRF1-dependent mechanism of IFN-β induction in virus-infected cells. Mapping of the promoter region of this functionally important gene in IFN-β induction made it apparent that IRF1 possesses a virus-inducible promoter (Miyamoto et al, 1988). Subsequently, IRF2 was identified to be binding to the same upstream regulatory *cis* element of type 1 IFN by cross hybridization with IRF-1 cDNA (Harada et al, 1989). Increases in both IRF1 and IRF2 mRNA levels

were observed in virus-infected cells, followed by IFN-β accumulation. cDNA analysis revealed that both IRF members contain a well-conserved DNA-binding domain at the N-terminus (Harada et al, 1989). However, while IRF1 has been shown to possess a transcription activation domain at the C-terminus, the C-terminus domain of IRF2 appeared to have significant differences. Co-transfection assays carried out using IRF1- and IRF2-encoding plasmids with another construct carrying the IFN-β promoter and a reporter gene confirmed their hypothesis that IRF2 competitively binds to IFN-β gene regulatory sequences to repress IRF1 activation (Harada et al, 1989). Interestingly, although both IRF1 and IRF2 genes were found to be IFN-β-inducible, IRF1 mRNA was rapidly induced within 1 h after IFN-β addition, while IRF2 mRNA induction peaked more slowly at 4 h (Harada et al, 1989). The delay in IRF2 induction suggests that it plays a critical role in reversing IRF1-mediated induction of type 1 IFN, which the authors postulated could make the gene promoter regions accessible for subsequent activation by other factors (Harada et al, 1989). However, the model of the IRF1/IRF2 paradigm being the exclusive IRFs mediating the type 1 IFN activation was disputed by the finding that disruption of IRF1 in mice did not impair the induction of type 1 IFN by virus infection (Matsuyama et al, 1993).

Indeed, other IRFs essential for the induction of IFN-inducible genes were soon discovered. IFN-stimulated response elements (ISRE) on the promoter region of ISGs, which are similar to the *cis* regulatory promoter regions of type 1 IFN, were discovered to be induced through binding of a constitutively expressed factor, IRF3 (Au et al, 1995). Relative mRNA levels of IRF3 did not increase in virus infection or type 1 IFN treatment. However, the over-expression of IRF3 resulted in the expression of ISG15, an IFN-stimulated gene, and this observation hinted at the complexity and post-transcriptional regulation imposed by different members of IRFs on type 1 IFN

induction (Au et al, 1995). Later, it was found through co-immunoprecipitation experiments that IRF3 interacts with CREB-binding protein (CBP)/p300 co-activators to form a double-stranded RNA-activated transcription factor 1 (DRAF-1) complex which then binds to the ISRE of type 1 interferons and certain ISGs (Weaver et al, 1998). The evidence of the phospho-regulation of an IRF member was first presented when the DNA-binding activity of the DRAF-1 complex was found to be abrogated by phosphatases. Correspondingly, serine phosphorylation of IRF3 was observed following a virus infection (Weaver et al, 1998). The essential role of IRF3 in type 1 IFN induction through toll-like receptor (TLR) signaling was corroborated in studies showing that type 1 IFN could not be induced in $IRF3^{-/-}$ mice or IRF3-deficient mouse embryonic fibroblast (MEF) cells (Sato et al, 2000; Sakaguchi et al, 2003).

Binding of type 1 IFN to its receptor results in the formation of a transcriptional activator known as IFN-stimulated gene factor 3 (ISGF3), which consists of IRF9, signal transducer and activator of transcription 1 (STAT1) and STAT2 (Honda and Taniguchi, 2006). Among the ISGs stimulated by virus infection through ISGF3 is an IRF highly analogous to IRF3, IRF7 (Zhang and Pagano, 1997). IRF7 induction was reported to be impaired in the absence of STAT1 (Marié et al, 1998). Unlike IRF3 which is constitutively expressed in small amounts, IRF7 is expressed in small amounts but strongly induced downstream by type 1 IFNs (Honda and Taniguchi, 2006). The distinct roles of IRF7 and IRF3 in response to viruses were established with observations that IRF3, but not IRF7, is necessary to initiate IFN-β induction. Instead, IRF7 participates in a positive feedback mechanism whereby it binds the ISRE of the type 1 IFNs to induce effective amounts of both IFN-α and IFN-β (Sato et al, 2000; Sakaguchi et al, 2003; Honda and Taniguchi, 2006). These findings led to the idea of a biphasic type 1 IFN induction model, in which IRF3 is mainly

responsible for the early phase induction of IFN-β while the late phase is initiated through IFN-β-dependent IRF7 induction and allows for the production of the complete set of type 1 IFNs (both IFN-α and IFN-β) (Sato et al, 2000; Honda and Taniguchi, 2006).

The family of IRFs includes IRF1, IRF2, IRF3, IRF4, IRF5, IRF6, IRF7, IRF8 and IRF9 (Taniguchi et al, 2001; Paun and Pitha, 2007). They have many gene targets with roles in antiviral and immunomodulatory functions. Among the IRF family members, IRF3 and IRF7 are of particular interest in the type 1 IFN pathway as IRF3 activation is crucial for the induction of IFN-β and IRF7 activation enhances the IFN-α/β response (Fig. 1.1).

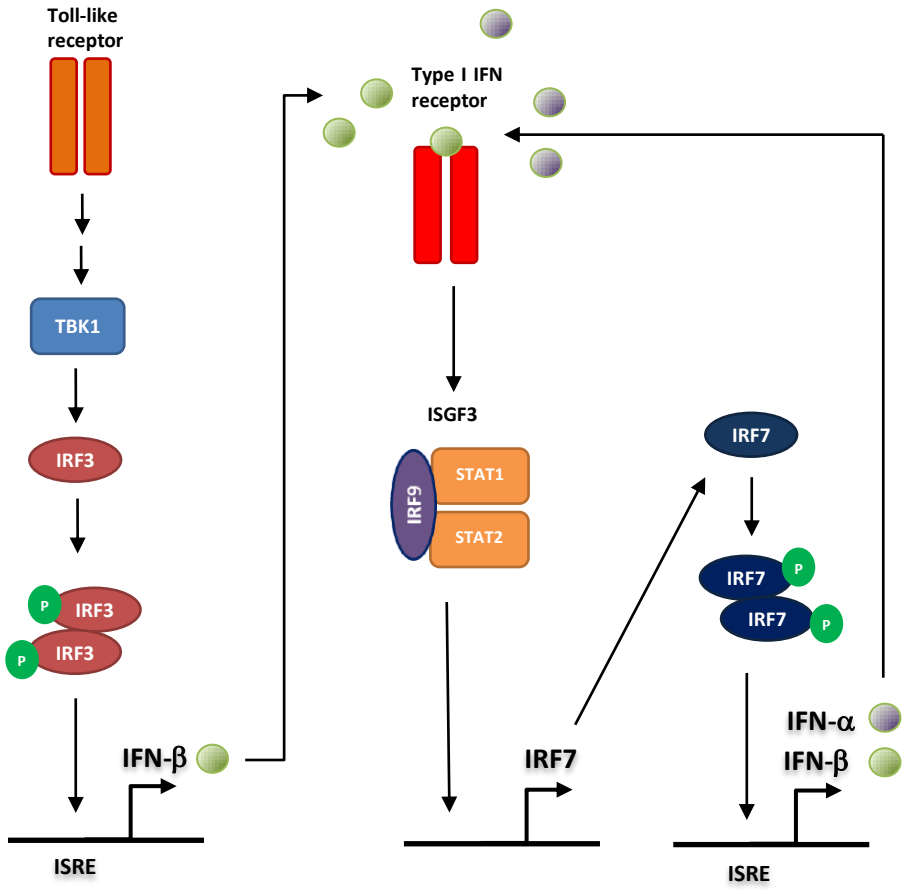

Fig 1.1 The involvement of IRF3 and IRF7 in the Type I IFN response.

1.2 The link between IRFs and tumour suppression

There is considerable knowledge on how IRFs mediate the host defense against viral infections. Apart from having antiviral functions, another critical facet of the IRF family members' roles in host defense is that they have roles in genotoxic stress-triggered signal transduction pathways (Taniguchi et al, 2001). The link between IRF pathways and tumour suppression was first realized when IRF1 over-expression was found to possess tumour-suppressive activities, while IRF2 enhanced the tumorigenicity of mouse fibroblast cells (Harada et al, 1993). Further evidence of such tumour suppression led to investigations into the role of IRFs in activating the DNA damage response and apoptosis, both of which are barriers to tumorigenesis. Using $IRF1^{-/-}$ mice, Tamura et al was the first to show that IRF1 is essential for radiation-induced apoptosis in T lymphocytes (Tamura et al, 1995). The same study also showed that the cell death gene interleukin-1β converting enzyme (ICE) (now known as Caspase 1) is induced through an IRF1-dependent mechanism. Shortly after, another study utilizing irradiation of $IRF1^{-/-}$ mouse embryonic fibroblasts (MEF) cells elucidated the p53-independent induction of cyclin dependent kinase (CDK) inhibitor p21 (WAF1/CIP1) by IRF1 (Tanaka et al, 1996). The observation led to the suggestion that IRF1, by switching on genes in the p53-independent arm, converge synergistically with p53-mediated mechanisms to arrest cell cycle through the common target gene p21 (Tanaka et al, 1996).

This level at which p53 and IRF1 cooperate in response to genotoxic stress was postulated to be at the G1-checkpoint pathway level (Tanaka et al, 1996). The DNA damage sensor ATM (ataxia telangiectasia, mutated) is well known to play a crucial role in the regulation of p53 in the event of DNA strand breaks (Barlow et al, 1997; Banin et al, 1998; Bakkenist and Kastan, 2003). The possibility of ATM also having a

regulatory role in signaling the presence of genotoxic stress to IRF1 was intriguing. This was recapitulated when fibroblasts from patients with ataxia telagienctasia (AT), which are defective in the ATM signaling pathway, were observed not to be able to induce IRF1 mRNA transcription, while exhibiting no defects in IRF1 induction through TLR-mediated signaling (Pamment et al, 2002). Reconstitution of ATM in these fibroblasts restored IRF1 induction in response to radiation-induced genotoxic stress, highlighting the ATM-dependent mechanism of IRF1 induction in response to DNA damage (Pamment et al, 2002).

As a central mediator of the innate host defense mechanism, IRF3 is posited to have roles in tumour suppression as well. In fact, one defense mechanism against viral propagation in host cells is the induction of apoptosis of infected cells. Sendai virus- and NDV virus-induced apoptosis was inhibited when dominant mutants of IRF3 were expressed in host cells (Heylbroeck et al, 2000; Weaver et al, 2001). Interestingly, data from Weaver et al suggests that, through using U3A cells that are deficient in IFN signaling and a truncated p53 dominant mutant, IRF3-induced apoptosis may be both IFN- and p53-independent. Perhaps the most functional revelation of the tumour suppressive role of IRF3 came in a study whereby overexpression of IRF3 in B16 melanoma cells suppressed their growth when transplanted into mice (Duguay et al, 2002).

In addition to IRFs and IFN-independent tumour suppression, other studies have suggested that IFN-α/β is directly integrated into the p53 tumour suppression responses. When MEF cells were stimulated by IFN-β, an increase in p53 levels could be observed (Takaoka et al, 2003). The authors subsequently found ISRE-similar sequences in p53 and binding of IRF9 to these sequences was discovered through chromatin immunoprecipitation assays (ChIP). The strong anti-oncogenic effect of

IFN-β was demonstrated in its suppression of induced transformation of MEF cells by the oncogene E6 (of the human papilloma virus) in a dose-dependent manner (Takaoka et al, 2003). Moreover, IFN-β-treated cancer cells were more susceptible to apoptosis in response to sub-optimal doses of DNA damage-inducing chemotherapeutic agent 5-fluorouracil (5-FU), indicating that IFN-β can elevate p53 levels to make the cells sensitive to DNA damage (Takaoka et al, 2003).

1.3 The DNA damage response is a barrier against tumorigenesis

The maintenance of genome integrity is crucial for the function and survival of all organisms. DNA aberrations can arise from the constant assault of intrinsic and extrinsic environmental stresses in the form of genotoxic agents, physiological processes such as DNA mismatches during DNA replication and the intrinsic biochemical instability of the DNA itself (Lindahl, 1993). DNA damage can also be caused by reactive oxygen species and nitrogen compounds produced by neutrophils and macrophages at sites of inflammation (Kawanishi et al¸ 2006). These DNA lesions or aberrations can block transcription and genome replication, and if not repaired, can lead to mutations or large-scale genome aberrations that threaten cell or organism viability. As such, organisms have evolved complex DNA damage responses (DDR) to combat the deleterious effects of DNA damage (Zhou and Elledge, 2000). Upon sensing damage to the DNA or stalled replication, sensor proteins recruit and activate checkpoint proteins to arrest the cell cycle to allow for initiation of DNA repair (Zhou and Elledge, 2000).

The key DDR sensor proteins are the protein kinases ATM and ATR (ATM- and Rad3-related). Single-stranded breaks (SSBs) in the DNA result in sections of ssDNA in the genome, which is then coated by replication protein A (RPA), a ssDNA-binding

protein complex. RPA recruits ATR to the site of damage while ATM is recruited by double-stranded breaks (DSBs) (Shiloh, 2003; Zou and Elledge, 2003). Some of the best-known respective targets of ATM and ATR are checkpoint signaling kinases 2 and 1 (CHK2/CHK1), which are kinases that regulate cell cycle progression (Falck et al, 2001; Zhao and Piwnica-Worms, 2001). Both CHK2 and CHK1 reduce the activity of cyclin-dependent kinases (CDKs). CDK2 drives the cell cycle G1–S transition and through the S phase, while CDK1 drives the transition of the G2 phase into mitosis (Falck et al, 2001; Zhao and Piwnica-Worms, 2001). Therefore, the CHK1/2-mediated inhibition of CDKs arrests the cell cycle at G1-S, intra-S and G2-M checkpoints. Concomitantly, ATM/ATR enhances DNA repair by activating various DNA repair proteins and co-factors both transcriptionally and post-transcriptionally (Huen and Chen, 2008).

Tumorigenesis is a process that selects for genetic mutations and epigenetic changes in somatic cells which allow them to bypass cell cycle arrest and apoptotic mechanisms (Bartkova et al, 2005). One important protein that is frequently mutated in cells to allow evasion of such mechanisms is p53. The accumulation and activation of p53 is regulated by ATM/ATR and their downstream kinases CHK2/CHK1. Following DNA damage or stalled replication, the Ser-15 residue of p53 is directly phosphorylated by ATM/ATR and the activated p53 then upregulates target genes involved in the DDR (Dumaz and Meek, 1999), one of which is p21, a CDK inhibitor which suppresses CDK2 kinase activity thereby resulting in G1 arrest (Wade Harper et al, 1993; Chen et al, 1995). ATM/ATR also phosphorylates Ser-20 of p53, blocking the interaction between p53 and its inhibitor MDM2 to aid its accumulation in the cell nucleus (Hirao et al, 2000). In severe cases of irreparable DNA damage, p53 functions to induce apoptosis by activating genes in both the mitochondrial and CD95-

FasL apoptotic pathways (Kastan et al, 1991; Lowe et al, 1993; Bennett et al, 1998; Chipuk et al, 2003; Mihara et al, 2003) Thus, the loss of p53 function results in the loss of the DDR and the apoptotic machinery, which is major step in which the cell can overcome its intrinsic safeguard mechanism to stop proliferating with DNA damage – an essential step in tumorigenesis. Indeed, the functional inactivation of p53 has been observed in 50% of all human cancers (Hanahan and Weinberg, 2000). Activation of oncogenes and the deregulation of DDR pathways and tumour suppressors such as p53 lead to the accumulation of DNA damage, notably DSBs, which contributes to continuous genomic instability (Halazonetis et al, 2009). Genomic instability is another fundamental feature of cancer which is observed in the vast majority of solid human tumours (Lengauer et al, 1998). Furthermore, genomic stability in the form of chromosomal instability (CIN) can arise when telomeres in preneoplastic tissues become critically short and prone to chromosomal fusions (Maser and DePinho, 2002).

The importance of the DDR as a barrier against tumorigenesis is evidenced by studies on human tumour samples that show the activation of the DDR in early precancerous lesions (Bartkova et al, 2005; Gorgoulis et al, 2005). These studies suggest that DNA damage checkpoints are constitutively active in the early stages of human tumorigenesis, well before p53 mutations arise. This barrier against tumour progression creates selection pressure for precancerous cells to deactivate components of the DDR through inactivating mutations or epigenetic inactivation, and might account for the high frequency of p53 mutations observed in human cancers. Studies on transgenic mice models have also identified functional deficiency in various DDR components and mutations in the genes of the pathway result in tumour onset at varying ages (Ishikawa et al, 2004). It is likely that the mutational inactivation of the

DDR pathway genes results in tumour cell survival and proliferation despite wide-spread mutations and genomic or chromosomal instability.

1.4 The NKG2D ligands and their upregulation by the DNA damage response

Due to the prevalence of DNA damage and the complexity of cellular signaling networks within the cell, it is inevitable that cross-talks and integration of the DDR with other signaling pathways occur. The involvement of the DDR in diverse biological and disease settings (Jackson and Bartek, 2009) and the purported link between innate immune pathway mediators (e.g. IRFs) and tumour suppression pathways exemplify such integration, especially in the context of natural immunity against cancer. The ability of the immune system to detect and eliminate nascent tumours and immune evasion of tumour cells as a necessary step in tumour establishment is a concept known and debated for decades (Swann and Smyth, 2007). Findings in humans and animal cancer models in recent years now offer compelling evidence that transformed cells that have escaped intrinsic barriers against tumorigenesis face extrinsic suppression by the immune system (Swann and Smyth, 2007). The process by which the immune system can 'sense' precancerous or cancer cells on the basis of tumour-specific antigens or specific surface molecules and target them for destruction before they can proliferate is termed tumour immunosurveillance.

As DNA damage and the activation of DDR in precancerous or nascent tumours constitute an intrinsic barrier against tumorigenesis, it was of interest if the DDR can also induce the expression of surface molecules that can be recognized by the immune system. Indeed, it was observed that ligands for the activating immune receptor NKG2D (NK group 2, member D) were upregulated in response to DNA damage (Gasser et al, 2005). The NKG2D receptor is expressed on all natural killer (NK) cells, cytotoxic $CD8^+$ T cells and tested γδ T cells in humans. In contrast, NKG2D

expression on mice $CD8^+$ T cells is limited to activated $CD8^+$ T cells and only certain subsets of γδ T cells in mice express NKG2D. All mouse NK cells do, however, express NKG2D, as do activated murine macrophages and NKT cells (Fig. 1.2) (Raulet, 2003). The NKG2D receptor is a co-activating receptor for T cell activation in the adaptive immune system and of particular interest, one of the activating receptors required for NK cell cytotoxicity (Gasser and Raulet, 2006).

Cell type	Cell-surface expression pattern	
	Mouse	Human
NK cells	~100%	~100%
$CD8^+$ αβ T cells	Before activation: absent After activation: ~100% Antigen-specific memory cells: ~100%	Before activation: ~100% After activation: ~100% Antigen-specific memory cells: ~100%
$CD4^+$ αβ T cells (conventional)	Rare or absent	Normally absent, upregulated in rheumatoid arthritis patients
$NK1.1^+$ T cells	~70% positive	N. D.
γδ T cells	Splenic γδ T cells: ~25% Intestinal intraepithelial γδ cells: absent Dendritic epidermal γδ T cells: ~100%	Peripheral blood γδ T cells: ~100% Intestinal intraepithelial γδ cells: ~100%
Macrophages	Before activation: absent After activation (with LPS, IFN-α/β or IFN-γ): ~100%	Absent on monocytes and macrophages

IFN, interferon; LPS lipopolysaccharide; N. D. not determined; NK, natural killer.

Fig 1.2 Expression of NKG2D by immune cells. Adapted from Raulet, 2003.

The serendipitous discovery of NK cells was made during the course of studies intended to find cytotoxic T cells in cancer patients and animal tumour models (Herberman 1987). The discrete subpopulation of granular lymphocytes in normal patients with the capability to kill tumour cells *ex vivo* become known as 'natural killer' cells for their spontaneous cytotoxicity against tumour cells and virus-infected cells (Herberman 1987). Spontaneous cytotoxicity against tumour cells represents the innate arm of tumour immunosurveillance, and hence NK cells are the crucial frontline first-responder to the danger posed by cellular transformation and tumorigenesis. The NKG2D ligands that activate the NKG2D receptors on NK cells are non-classical major histocompatibility complex (MHC) class I molecules and they

can be distinctly grouped into MIC (MHC class I chain-related) and RAET1 (retinoic acid early transcript 1) gene families. The MIC family members, MICA (MHC Class I polypeptide-related sequence A) and MICB (MHC Class I polypeptide-related sequence B), are encoded in the human major histocompatibility complex (Bauer et al, 1999; Raulet, 2003). MICA or MICB are expressed on many tumour cell lines and primary tumours of epithelial origins. However in normal human cells, MICA or MICB are expressed only by intestinal epithelial cells, most likely as a consequence of stimulation by the bacterial flora (Groh et al, 1996). No MIC homologs have been found in the mouse genome so far. The human RAET1 family, also known as UL16-binding proteins (ULBPs), consists of genes with a similar expression pattern in tumour or diseased cells. The mouse Raet1 genes can be further divided into Rae1, H60 and Mult1 subfamilies that share little homology but are structurally similar. The Rae1 subfamily consists of highly related isoforms Rae1α - Rae1ε encoded by different genes (Raulet, 2003).

Gasser et al (2005) has found that DNA damage-inducing agents or replication-inhibiting agents induce the expression of the mouse NKG2D ligands of the Raet1 family and the induction of Raet1 is inhibited when the DDR pathway proteins ATM/ATR are pharmacologically inhibited. On tumour cells that express Rae1 constitutively, blocking the function of ATM, ATR or CHK1 kinases led to substantial decreases in surface expression (Gasser et al, 2005). Their findings support the idea that persistent DNA damage in these tumour cells maintains constitutive NKG2D ligand expression. More intriguingly, they discovered that $p53^{-/-}$ cells are able to upregulate NKG2D ligands in response to genotoxic agents (Gasser et al, 2005). These findings suggest that despite being deficient in a major component of the DDR, NKG2D-mediated tumour immunosurveillance is not compromised (Gasser et al,

2005). It is plausible that genomic instability is manifested through the accumulation of SSB/DSBs in the absence of p53 and ATM/ATR is recruited to sites of DNA damage. Though unable to initiate the apoptotic signaling cascade, ATM/ATR is able to induce the expression of NKG2D ligands through a p53-independent mechanism.

1.5 NKG2D ligand-mediated activation of NK cells is an important tumour response

One general mechanism by which tumour cells evade $CD8^+$ T cell-mediated immunity is through the downregulation of MHC class I molecules (Garrido et al, 1993; Algarra et al, 2004). However, many inhibitory receptors expressed by NK cells recognize MHC class I molecules and this reduction of inhibitory signaling, coupled with the stimulation of activating receptors such as NKG2D, contributes to the net activating signals received by the NK cells and increases the susceptibility of the tumour cells to NK cell lysis (Gasser and Raulet, 2006). A number of studies have highlighted the importance of the NKG2D receptor for the control of tumour growth and metastases. One study showed that the expression and surface density of MICA and ULBP on human tumours of different origin is positively correlated with the capability of NK cells to kill through NKG2D-mediated mechanisms (Pende et al, 2002). However, the fact that these tumour cells naturally express NKG2D ligands when their expression is limiting tumour growth suggests that tumour cells often express insufficient levels of NKG2D ligands to stimulate immune rejection, possibly due to the selection of ligand expression levels on the tumours by the immune system *in vivo* as the tumour evolves. Evidence pointing to immune modulation of the NKG2D ligands on tumour cells can be inferred in mice where emerging sarcomas in perforin-deficient mice (hence dysfunctional in cell-mediated cytotoxicity) were Rae1-expressing and proven to be immunogenic when transferred into wild type mice

with full NK cell effector functions (Smyth et al, 2004). It was also observed that the neutralization of NKG2D could enhance the susceptibility of mice to carcinogen-induced fibrosarcoma. (Smyth et al, 2004). Human tumours have also been shown to evolve mechanisms to produce high levels of a soluble isoform of MICA that causes desensitization of the NKG2D receptor on T cells (Groh et al, 2002). Before Gasser et al made the observation that Raet1 ligands could be induced by genotoxic drugs (Gasser et al, 2005), attempts had already been made to reintroduce immunogenicity in melanoma tumour cell lines. B16-BL6 melanoma cell line, one of the most tumorigenic and least immunogenic tumour cell lines used frequently for subcutaneous tumour transplantation experiments, lacks expression of NKG2D ligands. But when it was transfected with Rae1 or H60, the melanoma cells were efficiently rejected by syngeneic B6 mice (Diefenbach et al, 2001). Depletion of NK cells resulted in the loss of the capability of the mice to reject the melanomas (Diefenbach et al, 2001). These studies all provide firm evidence that the expression of NKG2D ligands and the activation of the NKG2D receptor are prerequisites for an effective anti-tumour response.

The understanding of the NKG2D receptor in anti-tumour responses has driven the development of novel cancer immunotherapeutic strategies using chimeric NKG2D receptors. Chimeric NKG2D-CD3ζ receptor-bearing T cells have been engineered to recognize and kill tumour cells in ovarian cancer mouse models through an MHC-independent manner. Furthermore, long-term surviving mice were able to overcome re-challenges with ovarian tumours in part through the expansion of non-modified tumour-specific T cells, showing that the adaptive response was also primed in this tumour-targeting strategy (Zhang et al, 2005; Barber et al, 2008). This approach has the potential to be applied to other tumours such as lymphomas, melanomas and

breast cancer. Another human chimeric NKG2D-Fc fusion protein that mediates complement-dependent lysis, antibody-dependent cellular cytotoxicity (ADCC) and effective opsonization of NKG2D ligand-expressing tumor cells was recently patented for future applications in human cancer therapy (Dranoff et al¸ WO 2010/080124 A9). The successful use of chimeric NKG2D receptors in mouse cancer models will open up a new avenue of exploratory research in the immunotherapy of cancers in human patients and again reiterates the importance of the NKG2D-NKG2D ligand signaling system in tumour control.

1.6 Rationale of study

Although there is evidence of ATM/ATR-mediated DNA damage signaling inducing the expression of NKG2D ligands, the underlying pathway that links the DDR to the upregulation of NKG2D ligands is not clear. The importance of the NKG2D ligands in tumour immunosurveillance necessitates the study of this pathway. Understanding this pathway could possibly result in development of combinatorial therapies with genotoxic agents that further increase the expression of NKG2D ligands on tumour cells. Interestingly, the effector functions of the ATM/ATR kinases in this pathway are p53-independent and given the broad specificity of ATM/ATR or their downstream kinases CHK2/CHK1, we propose that they have other hitherto unknown targets in other non-apoptotic or cell cycle-related pathways. The fact that the upregulation of NKG2D ligands on fibroblasts is observed at 16-18 hours after treatment with DNA damaging compounds hints at transcriptional regulation of NKG2D ligands (Gasser et al, 2005). The maintenance of NKG2D ligands on tumour cells was proposed to be through constitutive activation of the DDR (Gasser et al, 2005). Hence, it is likely that ATM/ATR constantly triggers a signaling cascade that culminates in the expression of NKG2D ligands. It was of our interest to verify if both

the genotoxic drug-induced upregulation of NKG2D ligands and the constitutive expression of NKG2D ligands in tumour cells share the same ATM/ATR-mediated pathway. The IRF family of transcription factors has diverse immunomodulatory roles apart from antiviral responses. The abovementioned tumour-suppressive roles of IRF3 is supported by a finding that IRF3 is activated and localized to the nucleus in response to DNA damaging agents (Kim et al, 1999). It would be of our interest to reproduce this observation using our own cellular models of NKG2D ligand induction and study if IRF3 is directly involved in the expression of NKG2D ligands.

CHAPTER 2: MATERIAL AND METHODS

2.1 Mice

2.1.1 Breeding

C57BL/6 mice were purchased from the Centre for Animal Resources at the National University of Singapore. The mice were bred and housed as described (Jamieson et al, 2002).

2.1.2 Isolation of TBK1$^{-/-}$IKKε$^{-/-}$ and TBK1$^{+/+}$IKKε$^{+/+}$ MEF cells

TBK1$^{-/-}$IKKε$^{-/-}$ and TBK1$^{+/+}$IKKε$^{+/+}$ mouse embryonic fibroblasts (MEF) cells were generated from knockout mice as described in (Ishii et al, 2006). BC2 cells were a generous gift by L.M. Corcoran (Corcoran et al, 1999). Yac-1 cells were purchased from ATCC (USA).

2.2 Transduction of cell cultures

2.2.1 Retroviral expression constructs

Mouse Irf3, Tbk1, Ikkε or Sike1 cDNA were subcloned adjacent to the cytomegalovirus promoter in the pMSCV2.2-IRES-GFP proviral vector (gift of W. Sha, University of California, Berkeley). Human chimeric constructs IRF3-EGFP and IRF3A7-EGFP were gifts of S. M. McWhirter (University of California, Berkeley). The chimeric constructs were subcloned into MSCV-IRES-GFP.

2.2.2 ShRNA retroviral constructs

Tbk1 shRNA (5'-GAAGCCGTCTGGTGCAATA-3'), Ikkε shRNA (5'-CACTTCATCTACAAACAGT-3') and Irf3 shRNA (5'-GCGGTTGGCTGTTGACAAT-3') target sequences were selected based on Dharmacon's siRNA Design Center algorithm (Dharmacon). Control shRNAs included scrambled versions of the Tbk1 shRNA (5'-GAAGCCGTGTGCTGCAATA-

3'), the Ikkε shRNA (5'-CACTTTATCCACAAACAGT-3') and the Irf3 shRNA (5'-GCGGTTCGTTGATGGCAAT-3'). The shRNAs constructs were subcloned in the MSCV/LTRmiR30-PIG vector (Open Biosystems, USA) according to manufacturer's instructions.

2.2.3 Packaging of expression murine retrovirus

Subcloned retroviral expression or shRNA retroviral constructs were co-transfected into confluent monolayers of HEK293T packaging cells seeded on 6-well plates with plasmids expressing mouse retroviral Gag, Pol and Env genes using TransFectin (Biorad, Singapore) according to manufacturer's instructions.

2.2.4 Retroviral transduction of cell cultures

2 mL of each MSCV retrovirus supernatant was collected from HEK293T cells, passed through 0.45 μM filters for spin infection of cell lines seeded in 6-well plates at 2000 r.p.m. and 34^{o}C for 90 min. The MEF cells, prior to spin infection, were seeded to 50 % confluency while 2 mL each of the suspension BC2 and Yac-1 cells were seeded at 100,000 cells/mL in 6-well plates. The infected cell lines were FACS-sorted for GFP-expressing cells 2 days after spin-infection.

2.3 Treatment of cell cultures

Aphidicolin, caffeine, CGK733, cytosine β-D-arabinofuranoside hydrochloride (Ara-C), DMSO and Poly I:C were purchased from Sigma-Aldrich (Singapore). InSolution^{TM} ATM Inhibitor was obtained from Calbiochem (USA). The murine TLR9 ligand ODN1585 and ODN1668 control were purchased from Invivogen (USA).

2.4 Flow Cytometry

Cells were stained for different NKG2D ligands using rat IgG antibodies against pan-Rae1, Rae1αβγ, Rae1βδ and Rae1ε (R&D Systems, USA) at 2.5 μg/mL, followed by secondary donkey anti-rat IgG antibodies-coupled to APC at 1 μg/mL (ebiosciences, USA). Stained cells were analyzed by multicolor flow cytometry using a FACSCalibur (BD Biosciences, USA) and FlowJo. 8.8.6 (Treestar, USA).

For intracellular phospho-flow cytometry staining, cells were fixed with 0.25% paraformaldehyde at 37°C for 10 min, followed by addition of -20°C methanol and incubation on ice for 20 min. After washing, cells were resuspended in 2% mouse serum and stained with 1/100 rabbit-anti-phospho-IRF3-Ser396 (Cell Signaling Technology, USA) and 1/25 Alexa-647-conjugated anti-phospho-TBK1-Ser172 antibodies (BD Biosciences, USA) followed by Alexa-488-coupled rabbit IgG-specific antibodies (Invitrogen, Singapore). Control cells were treated with 2 U/μl λ-phosphatase (NEB, USA) at 37°C for 90 min before staining.

2.5 Quantitative real-time PCR

Total RNA was isolated using the RNeasy kit (Qiagen, Singapore). 1 μg of total RNA was reverse transcribed with random hexamers using a Transcriptor First strand cDNA Synthesis Kit (Roche, Singapore). 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, Singapore). PCRs were performed in triplicates using the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Singapore). PCR thermocycling parameters were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 15 sec and 72°C for 1 min. All samples were normalized to the signal generated from the

housekeeping gene HPRT. The following primers were used: Hprt-5': tgggaggccatcacattgt, Hprt-3': gcttttccagtttcactaatgaca; Irf3-5': aggaacaatgggagttcgag, Irf3-3': cagtgtcatgtcagctgtgc; Ifn-β-5': aatttctccagcactgggtg, Ifn-β-3': tctcccacgtcaatctttcc; Ifn-α-5': tgacctcaaagcctgtgtgatg, Ifn-α-3': aagtatttcctcacagccagcag; Mx1-5': aaacctgatccgacttcacttcc, Mx1-3': tgatcgtcttcaaggtttccttgt, Pkr-5': ccaaagagaaaggcaggctcc, Pkr-3': ttcctccctccctcctccattc; IP-10-5': gctgcaactgcatccatatc, IP-10-3': tttcatcgtggcaatgatct; Ccl-5': accactccctgctgctttgc, Ccl5-3': cacacttggcggttccttcg; Isg15-5': tccatgacggtgtcagaact; Isg15-3': gaccagactggaaagggta; Isg54-5': gggcttcatccagcaacagc, Isg54-3': cctcctcacagtcaagagcagg; IfnαR-5': atgggcagtgtgacctttc, IfnαR-3': cccttcctctgctctgacac. Samples prepared without reverse-transcription served as negative control templates.

2.6 Confocal Microscopy

Treated BC2 and Yac-1 cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature, rinsed thrice with 1 X PBS and permeabilized using 0.2% Triton-X in PBS for 15 min at room temperature. Blocking was done using the staining buffer comprising of 1% BSA + 2% FCS in PBS for 30 min. The cells were then co-stained with 4.3 μg/mL anti-SIKE1 rabbit IgG (Sigma-Aldrich, Singapore) and 10 μg/mL anti-ATM (Ser1981) mouse IgG1κ (clone 10H11.E12, Millipore, Singapore) antibodies in staining buffer overnight at 4^{o}C, washed thrice with 1 X PBS and then co-stained with goat anti-rabbit IgG coupled to Dylite 649 and goat anti-mouse IgG1 coupled to Cy3 at 1/200 dilution (Jackson ImmunoResearch Laboratories, USA) for I hour on ice. For negative control staining, the cells were stained with anti-SIKE1 antibody pre-incubated with 25 μg/mL SIKE1 blocking

peptides for 30 min and mouse IgG1 isotype control at 1/100 dilution (eBiosciences, USA).

For IRF3 localization studies, FACs-sorted and treated BC2 cells or FACS-sorted Yac-1 cells expressing IRF3-GFP or IRF3A7-GFP were fixed with 4% paraformaldehyde for 10 min, rinsed once with with 1 X PBS and incubated in 70% ethanol for 1 h at -20^{o}C.

All prepared cells were centrifuged onto glass slides using cytospin funnels (Fisher Scientific, Singapore) and mounted with coverslips after addition of Vectashield® mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Images were examined with the Leica confocal TCS SP5 microscope and retrieved using the built-in LAS AF software (Leica Microsystems, Singapore). Pictures were further analyzed using Photoshop CS4 (Abode Systems, USA) or ImageJ.

2.7 Mouse IFN-β and IP-10 ELISA

BC2 cells were seeded at 0.75 x 10^6 cells/mL. The levels of mouse IFN-β in the culture medium of BC2 cells treated with DMSO, 10 μM Ara-C or 1 μg/mL Poly I:C for 24 h were determined using the Verikine™ ELISA kit (PBL InterferonSource, USA) and the levels of mouse IP-10 5 h, 10 h, 15 h and 20 h after treatment were determined using the Quantikine® ELISA kit (R&D Systems, USA).

CHAPTER 3: RESULTS

3.1 Rae1 expression is induced upon DNA damage

NKG2D ligands have been shown to be upregulated in non-tumour cell lines in response to genotoxic stress induced by chemotherapeutic drugs that activate the ATM/ATR DNA damage checkpoint pathway (Gasser et al, 2005). We started by looking for a tumour cell line which has a low basal level of NKG2D ligand expression. We found that BC2 cells, a murine B lymphoma cell line established from the Eμ-Myc mouse model (Corcoran et al, 1999), lack NKG2D ligand expression and hence, is a suitable tumour cell line model to study the induction of NKG2D ligands in response to DNA damage. Not surprisingly, upregulation of the Rae1 family of NKG2D ligands was observed upon DNA damage induced by drug treatment (Fig 3.1). In contrast, the T lymphoma cell line Yac-1, which is sensitive to NK cell lysis and frequently used as positive controls for NK cell killing assays, has a constitutive level of NKG2D ligand expression expected of many tumour cell lines due to the constitutive activation of the DNA damage response pathway (Fig 3.1).

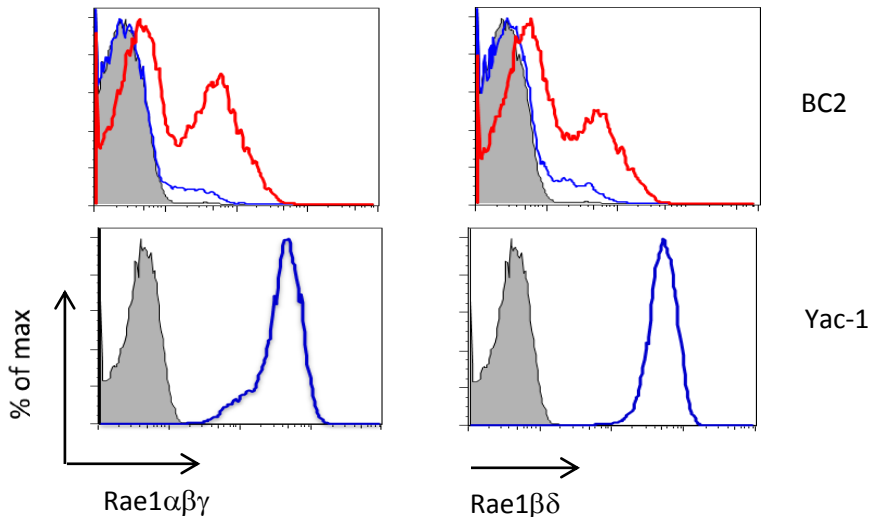

Fig. 3.1 Rae1 is induced upon DNA damage. BC2 cells were treated with DMSO (blue line) or 10 μM Ara-C (red line) for 18 hours and stained with antibodies against Rae1. Yac-1 cells were stained for Rae1 (blue) to show constitutive expression. Grey histograms of both cell types show isotype stainings.

3.2 IRF3 is required for Rae1 induction in response to DNA damage

3.2.1 IRF3 is phosphorylated in response to DNA damage

IRF3 has been shown to be phosphorylated and activated in response to DNA damage (Kim et al, 2005). We were interested in whether the upregulation of Rae1 expression during DNA damage correlates to IRF3 activation. The phosphorylation of IRF3 at Ser-396 (Ser-388 in murine IRF3) has been shown to be critical for its activation (Lin et al, 1998; Chen et al, 2008). Using flow cytometry, BC2 cells treated with DNA damaging agents Ara-C and aphidicolin increased expression of phosphorylated IRF3 (S388) from 15 hours onwards (Fig. 3.2A). IRF3 was constitutively phosphorylated at Ser-388 in Yac-1 cells and the specificity of the staining was verified by pretreating cells with λ-phosphatase (Fig. 3.2B).

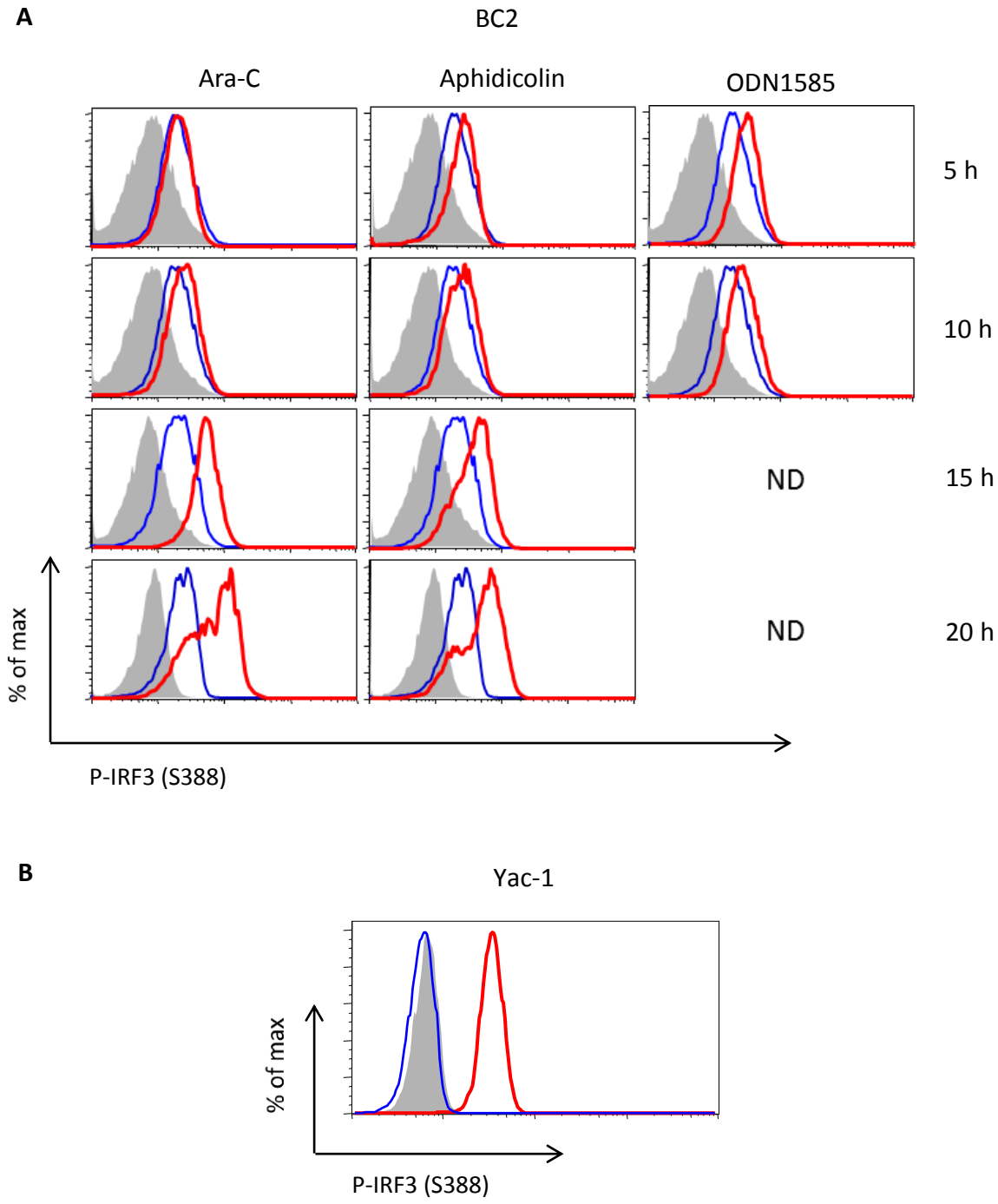

Fig. 3.2 IRF3 is phosphorylated in response to DNA damage. (A) BC2 treated with 10 μM Ara-C, 4 μM aphidicolin, 1 μg/mL ODN1585 (red line) or DMSO (blue line) for the indicated length of time were fixed, permeabilized and stained for phospho-specific IRF3 (S388). Isotype stainings of DMSO-treated cells are shown (filled histograms). **(B)** Phosphorylation at S388 of IRF3 was detected on Yac-1 cells (red line) using flow cytometry. Yac-1 cells pretreated with λ-phosphatase before staining were used as negative controls for the staining (blue line). The filled histogram shows isotype staining. *ND: Not determined.

3.2.2 IFR3 is translocated to the nucleus during DNA damage

The translocation of IRF3 into the nucleus is an important step in the activation of IRF3. To investigate if IRF3 translocation occurs in our cellular systems, we transduced BC2 and Yac-1 cells with a chimeric IRF3-GFP construct. Translocation of the chimeric IRF3-GFP fusion protein into the nucleus was observed in BC2 cells treated with Ara-C, as with BC2 cells treated with Poly I:C. Upon pre-treatment of the cells with the ATM/ATR inhibitor CGK 733, the translocation was not observed (Fig. 3.3). Yac-1 cells transduced with IRF3-GFP have constitutive activation of IRF3 and thus constitutive translocation of IRF3 into the nucleus (Fig. 3.3). Transduction of both BC2 and Yac-1 cells with a dominant negative mutant form of IRF3 in which the serine or threonine residues 385, 386, 396, 398, 402, 404 and 405, which are essential for IRF3 activation, had been replaced by alanine (IRF3A7-GFP) (Yoneyama et al, 1998; Yang et al, 2002) did not result in GFP translocation under any conditions (Fig. 3.3). DNA damage signaling thus resulted in the phosphorylation and nuclear translocation of IRF3, both of which are hallmarks of IRF3 activation. Our findings have also corroborated previous findings from another group that IRF3 activation occurs in response to DNA damage (Kim et al, 1999).

3.2.3 IRF3 target genes are induced upon DNA damage

Immunity to viral infection through the type 1 interferon (IFN-α/β) has been known to be mediated through the binding of IRF3 to IFN-β promoter regions, which results in the production of IFN-α/β. The secreted IFNs then activate a set of interferon-stimulated genes (ISGs) responsible for antiviral defence mechanisms (Juang et al, 1998; Sato et al, 1998). To verify if IRF3 transcriptionally activates the same set of genes during DNA damage response, the expression levels of several IRF3 target genes were determined. IRF3 target genes such as IFN-β, IP-10, CCL5 and

ISG15 were induced to a similar or greater degree by DNA damage as with Poly I:C (Fig. 3.4A). Increases in the secretion of IFN-β and IP-10 into the culture supernatants of Ara-C-treated BC2 cells were observed (Fig. 3.4B, C). However, the production of type 1 IFN itself was not responsible for Rae1 expression, as the addition of recombinant IFN-α or IFN-β failed to upregulate its expression (data not shown). Interestingly, the production of the chemokine IP-10 (CXCL10) in response to DNA damage was higher than that of during Poly I:C treatment (Fig. 3.4B, C).

3.2.4 Prediction of ISRE and NF-κB sites on promoter regions of Rae1

IRF3 and other members of the IRF family bind to the consensus ISRE sequence 5'-AANNGAAA-3' on the promoter regions of the genes they regulate (Fujii et al, 1999; Panne et al, 2004). We inspected the promoter sequence of Rae1α using MatInspector of the Genomatix software suite and further verified the sequence on NCBI database, and found a potential IRF-binding region upstream of the transcriptional start site (Fig. 3.5). The confirmation of the potential IRF binding site using computational methods supports the experimental evidence of IRF3 in regulating the expression of NKG2D ligands, but has to be verified using additional wet lab techniques such as Chromatin Immunoprecipitation (ChIP).

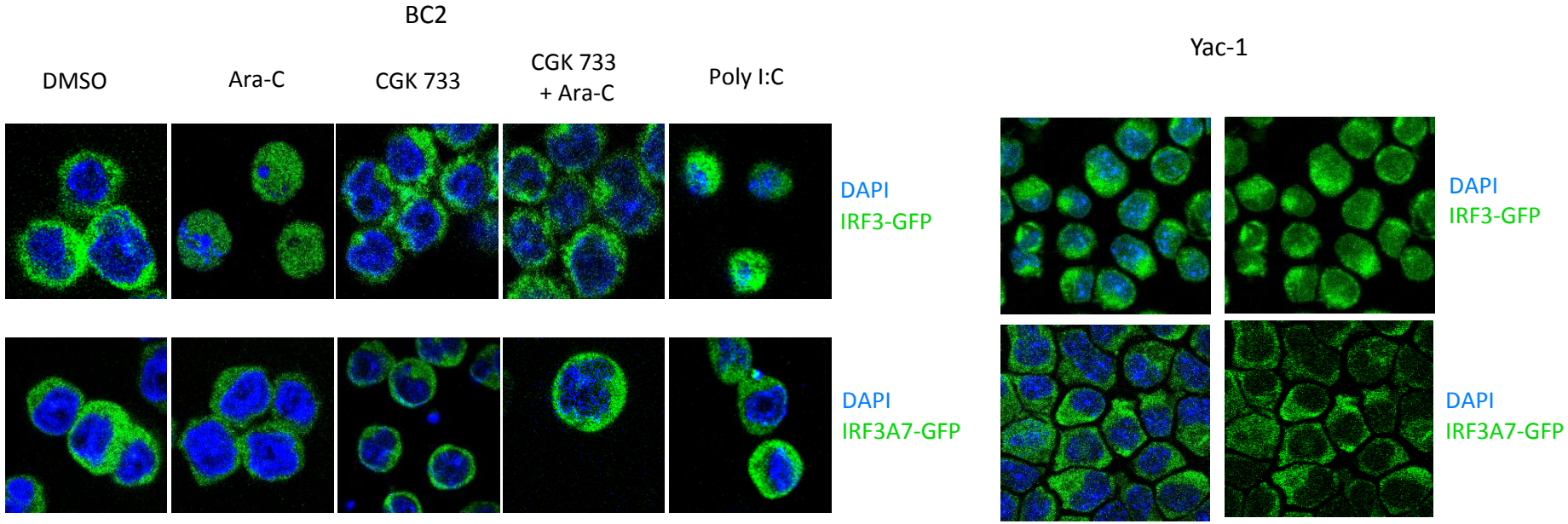

Fig. 3.3 Translocation of IRF3 into nuclei of BC2 and Yac-1 cells during DNA damage. BC2 and Yac-1 cells transduced with wildtype IRF3-GFP or dominant negative IRF3A7-GFP were FACS-sorted for GFP^+ cells. BC2 cells were then treated with DMSO, 10 μM Ara-C for 16 hours, 10 μM CGK 733 for 16 hours, CGK 733 (2h pre-treatment) + Ara-C (16h) or 10 μg/mL Poly I:C and stained with DAPI for observation using confocal microscopy. Yac-1 cells were sorted for GFP^+ populations, stained with DAPI and then mounted for confocal microscopy. The micrographs shown are representative of all images.

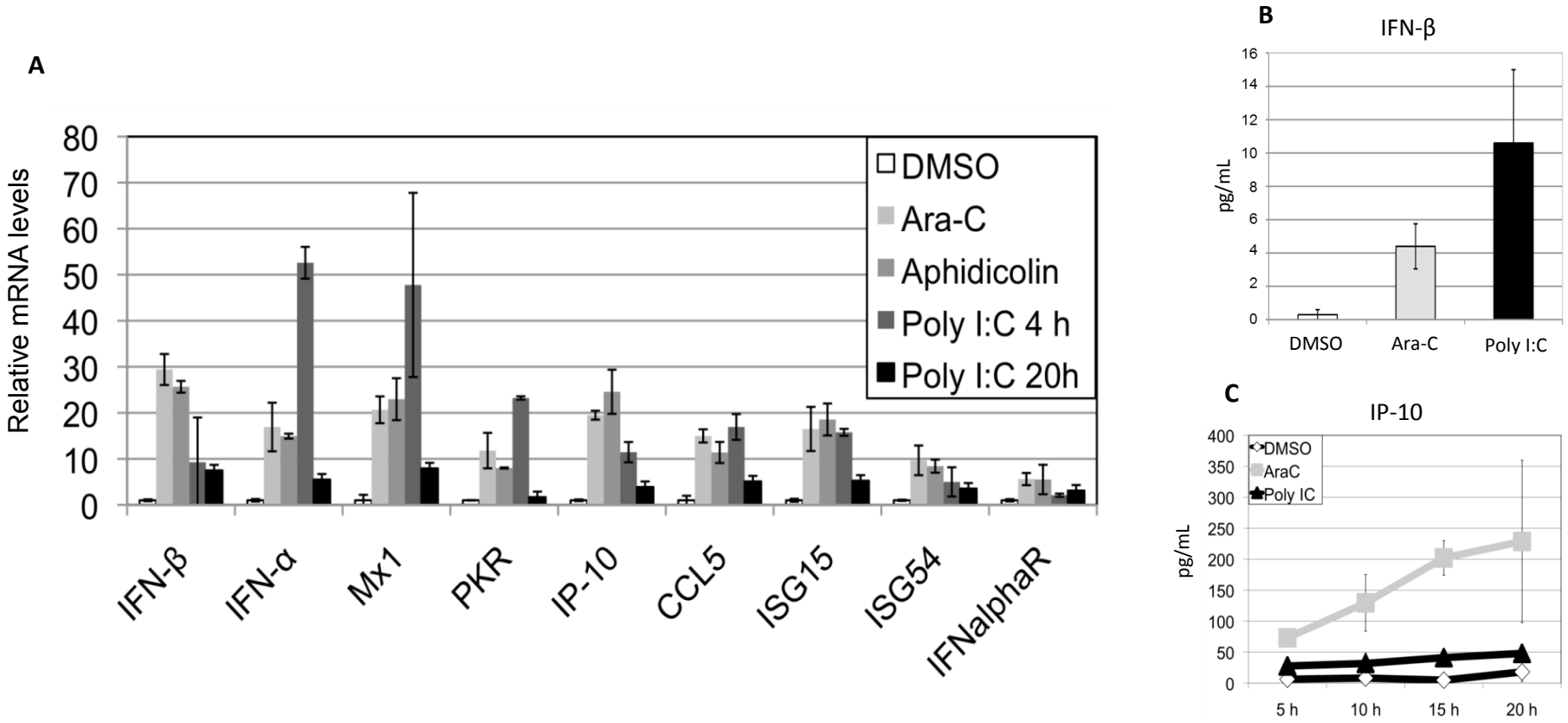

Fig. 3.4 Induction of IRF3 target genes by DNA damage. (A) BC2 cells were treated with DMSO (white bar), 10 µM Ara-C (light grey bar), 4 µM aphidicolin (grey bar), 1 µg/mL Poly I:C for 8 hours (dark grey bar), or 1 µg/mL Poly I:C for 20 hours (black bar). Relative mRNA levels of indicated genes were measured by real-time PCR. Means ± SD of 3 independent experiments normalized to DMSO treated cells are shown. **(B)** Levels of IFN-β in the culture supernatants of 0.75 x 10^6 BC2 cells/mL cells treated with DMSO, 10 µM Ara-C or 1 µg/mL Poly I:C for 24 h were determined by ELISA. **(C)** Levels of IP-10 in the culture supernatants of the same number of treated BC2 were measured at various time-points using ELISA.

A

-710 ACACAAGGTTAATCTCTGCCCTGTAAGCACACACACACACACACACACACACACACACAC
-646 ACACACACAAGGTTAATCTCTGCCCTGTAAGCACACACACACACACACACACACACACAC
-582 AAGGTTAATCTCTGCCCTGTAAGCACACACACACACACACACCTTTGCAAATATACAGTGCAGC
-518 TGTAAAACAGACTGGGGAAAAAAAAAAGTGACTCCCACATGATCTGCCAGGGACCTGGACTTT
-454 CCCTATGCAGAAGCCTGTTCTAC**AACAGAAA**GAAAAAGATTCAGTTATTTTTATCTCCATTGCA
-390 TGAGTTTTTTTTCTCTGTGTTTCTACACCATGTGTATGCAGTGTGTGCAGAGGTCAGAAACCTGC
-326 CTGGAATCCCCTGGAGCAGCAGTCACAGAAGGTTGTGAGCTGCTCGGTGAGTGCAGGAAATC
-262 CAACCTGGGTCCCAAAAGGGCAGTGTGTGCTCTTAACCTCTGAGCATCTTTTCAGGCCCCAGAA
-198 GAAGCTTCTAATTGGAAGAAAACTGCGACTGCAGAACTTTTCATGAGGTCTTAGATTCCCCTAG
-134 GACAGAGCTAAACAGAAAAGCACCAGCATTCCCTGGCGCGAGGGGGCAGAGGGGGTGGGGT
-70 TAGACGGAAGGGGCGGGGTTGGGAGGAAGCGGGAGGGTTTGGAGGGAAGAGGGGTGGCG
-6 GTGAGC**AGA**GCTATTGCGGAGGGTGAGGGTAACGGGATGGGGAGAAGGGACTAAGGAGAG
+58 TTGGGGGTGGGAAAGCAGTGGGACAACAAATATTTGAGGGTACAAAA

B

Fig. 3.5 ISRE binding site in Rae1α promoter region. The IRF-binding ISRE sequence with the known consensus 5'-AANNGAAA-3' (underlined, blue) is found upstream of the +1 transcriptional start site (TSS) (underlined, red). The promoter nucleotide sequence **(A)** and its graphical representation **(B)** are generated and analyzed using MatInspector of the Genomatix software suite.

3.3 TBK1 is necessary for Rae1 expression in response to DNA damage

3.3.1 TBK1 is phosphorylated in response to DNA damage

IRF3 is phosphorylated and activated by the two IKK-related serine/threonine kinases, TBK1 and IKKε (Fitzgerald et al, 2003; McWhirter et al, 2004; Yang et al, 2006), in the type 1 IFN pathway. We decided to investigate if TBK1 or IKKε is activated in response to DNA damage as well. Although the molecular mechanisms of TBK1 activation remains poorly defined, it is known that its kinase activity requires phosphorylation at Serine-172 (Shimada et al, 1999; Kishore et al, 2009). Phosphorylation of TBK1 at Ser-172 was detected by intracellular phospho-flow cytometry after treatment of BC2 cells with DNA damaging agents Ara-C or aphidicolin (Fig. 3.6A). A known activator of the TBK1 pathway, the synthetic dsRNA Poly I:C, induced phosphorylation at a shorter time interval after treatment. In agreement with this, TBK1 was also found to be constitutively phosphorylated in the Rae1-expressing Yac-1 cells (Fig. 3.6B). To verify the specificity of the P-TBK1 staining, cells pretreated with λ-phosphatase, which removes phosphate groups on serine, threonine and tyrosine, were used as negative controls. The phosphorylation of IKKε was not similarly assessed due to the lack of a phospho-specific IKKε antibody.

3.3.2 Drug-induced upregulation and constitutive expression of Rae1 is reduced by knockdown of TBK1

To further verify the role of TBK1 in regulating NKG2D ligand expression, we performed a knockdown of TBK1 in BC2 cells. Transduction of BC2 cells with TBK1 shRNA resulted in significantly lesser upregulation of Rae1 expression when the BC2 cells were treated with Ara-C, while transduction with IKKε shRNA had no effect on Rae1 expression (Fig. 3.7). This could be due to the compensatory effect of TBK1 on IKKε as there is partial redundancy observed between the two kinases which share

64% similarities in amino acid sequences and mediate similar downstream signaling events (Fitzgerald et al, 2003; Yamamoto et al, 2003). Constitutive expression of Rae1 also depends on TBK1 function, as Yac-1 cells transduced with TBK1 shRNA did show a considerable reduction of Rae1 expression (Fig. 3.8).

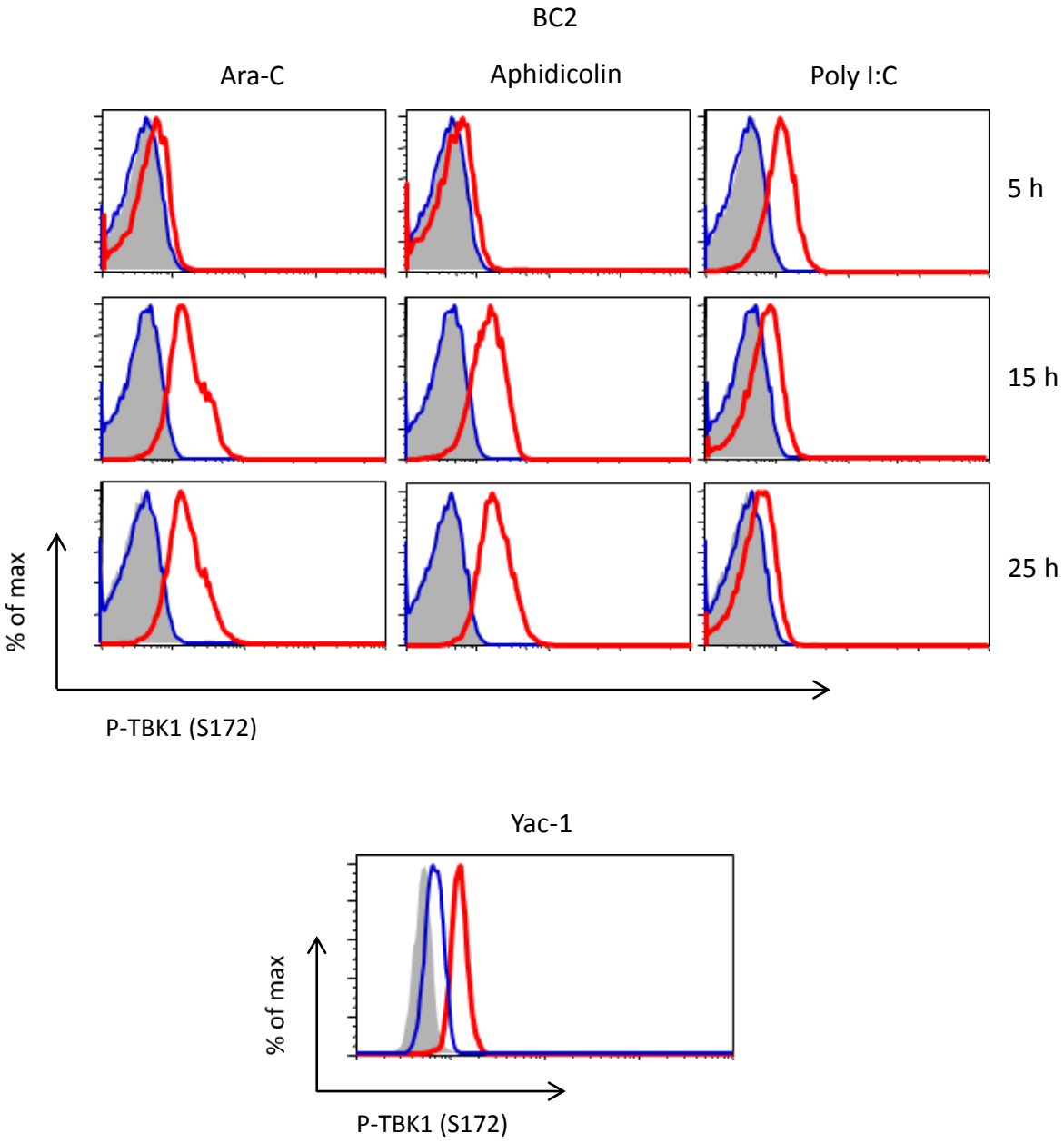

Fig. 3.6 TBK1 is phosphorylated in response to DNA damaging agents. (A) BC2 treated with 10 μM Ara-C, 4 μM aphidicolin, 1 μg/mL Poly I:C (red line) or DMSO (blue line) for the indicated length of time were fixed, permeabilized and stained for phospho-specific TBK1 (S172). Isotype stainings of DMSO-treated cells are shown (filled histograms). **(B)** Phosphorylation at S172 of TBK1 was detected on Yac-1 cells (red line) using flow cytometry. Yac-1 cells pretreated with λ-phosphatase before staining were used as negative controls for the staining (blue line). The filled histogram shows isotype staining.

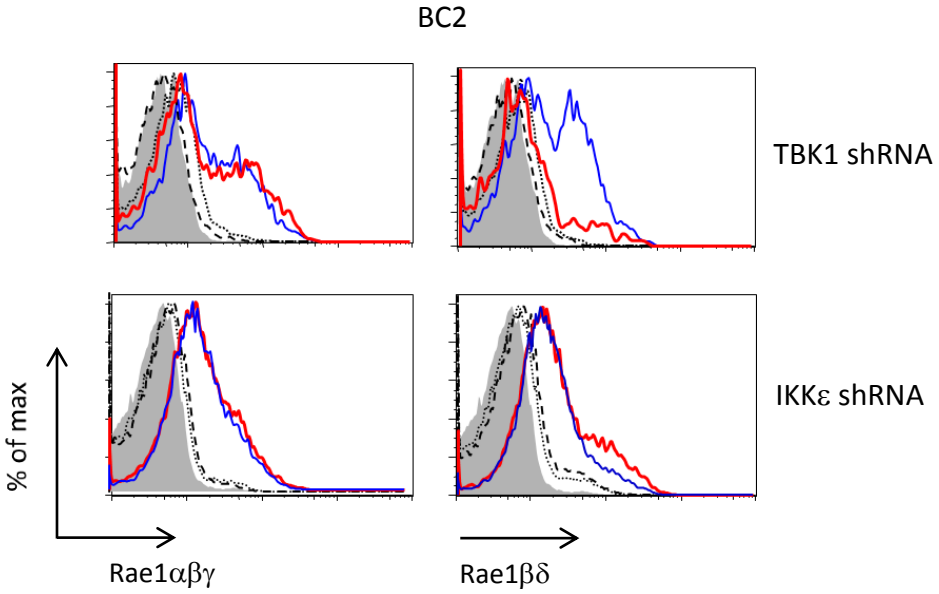

Fig. 3.7 Knockdown of TBK1 but not IKKε inhibits Rae1 upregulation in response to DNA damage. BC2 cells were transduced with retroviral vectors encoding TBK1 or IKKε shRNA with GFP (red line) or control shRNA with GFP (blue line), cultured for 5 days and treated with 10 μM Ara-C for 16 hours. Rae1 expression was determined by gating on transduced (GFP^+) cells. DMSO-treated cells expressing TBK1 or IKKε shRNA (dashed line) or control shRNA (dotted line) and Ara-C-treated cells with isotype staining (filled histogram) are shown.

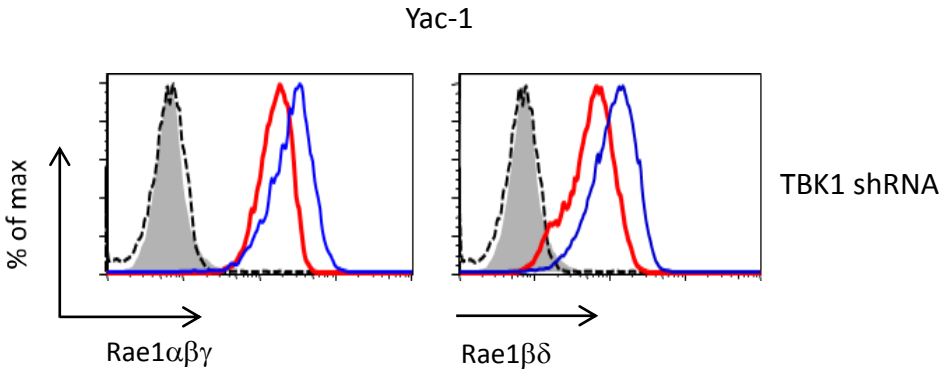

Fig. 3.8 Knockdown of TBK1 reduced constitutive Rae1 expression in Yac-1 cells. Yac-1 cells were transduced with retroviral vectors encoding TBK1 shRNA with GFP (red line) or control shRNA with GFP (blue line), cultured for 5 days and stained for Rae1 expression by gating on transduced (GFP^+) cells. Isotype stainings of TBK1-specific shRNA (dashed line) and control shRNA (filled histogram) are shown.

3.3.3 TBK1 inhibitor Sike1 inhibits DNA damage-mediated upregulation of Rae1

SIKE1 (Suppressor of IKKε) was discovered to be an inhibitor of TBK1/IKKε and thus a suppressor of TLR3- and virus-initiated IRF3 activation pathways (Huang et al, 2005). To investigate if SIKE1 is involved in the regulation of Rae1, BC2 cells were transduced with mouse Sike1 and treated with Ara-C. Rae1 induction was reduced in BC2 cells overexpressing Sike1 during DNA damage, in contrast with cells transduced with an empty control plasmid (Fig. 3.9). The constitutive expression of Rae1 on Yac-1 cells was also reduced by Sike1 overexpression (Fig. 3.10), suggesting that both drug-induced and replicative stress-induced genotoxic stimuli act through TBK1-mediated pathways to induce NKG2D ligands.

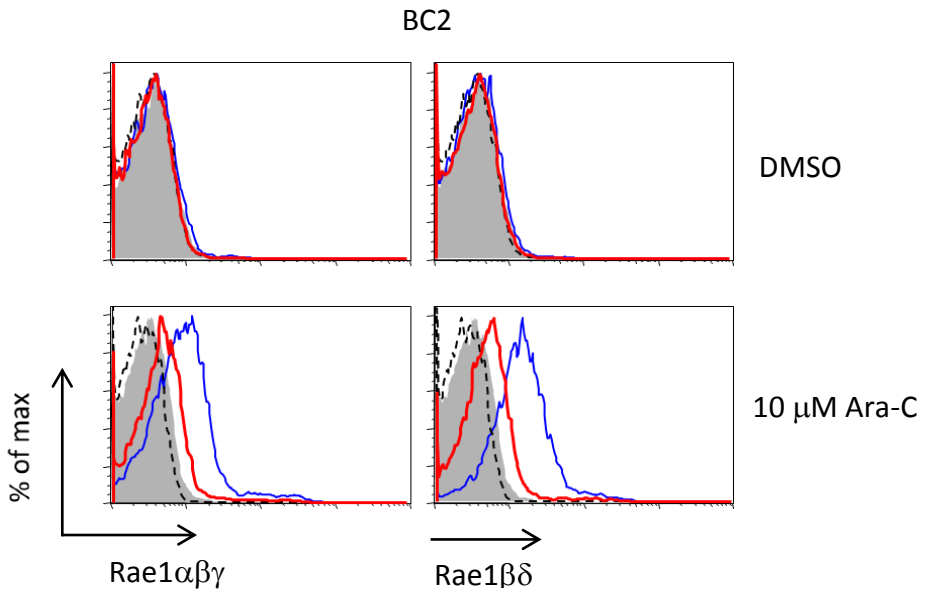

Fig. 3.9 Upregulation of Rae1 is abrogated by Sike1 overexpression. BC2 cells transduced with MSCV-Sike1-IRES-GFP (red) or control MSCV-IRES-GFP (blue) were sorted for GFP^+ transductants, cultured for 7 days and treated with DMSO or 10 μM Ara-C for 18 hours. Isotype control stainings for Sike1- (filled histogram) or control plasmid-transduced (dashed lines) cells are shown.

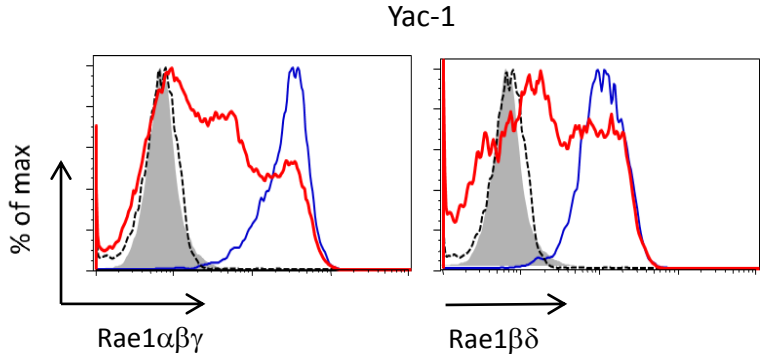

Fig. 3.10 Constitutive expression of Rae1 is reduced by Sike1 overexpression. Yac-1 cells transduced with MSCV-Sike1-IRES-GFP (red) or control MSCV-IRES-GFP (blue) were sorted for GFP^+ transductants, cultured for 7 days and stained for Rae1 expression. Isotype control stainings for Sike1- (filled histogram) or control plasmid-transduced (dashed lines) cells are shown.

3.3.4 Reconstitution of TBK1 in $TBK1^{-/-}$ $IKK\varepsilon^{-/-}$ MEF cells induces NKG2D ligand expression

To analyze if TBK1 is sufficient to induce the expression of NKG2D ligands, TBK1- and IKKε-deficient mouse embryonic fibroblast (MEF) cells were transduced with TBK1- and IKKε-encoding retroviral vectors (Matsui et al, 2006). Reconstitution of TBK1 or IKKε in TBK1/IKKε-deficient MEF cells induces the expression of NKG2D ligands (Fig. 3.11). Expression of TBK1 or IKKε restored the capacity of MEF cells to further upregulate NKG2D expression in response to DNA damage (Fig. 3.11).

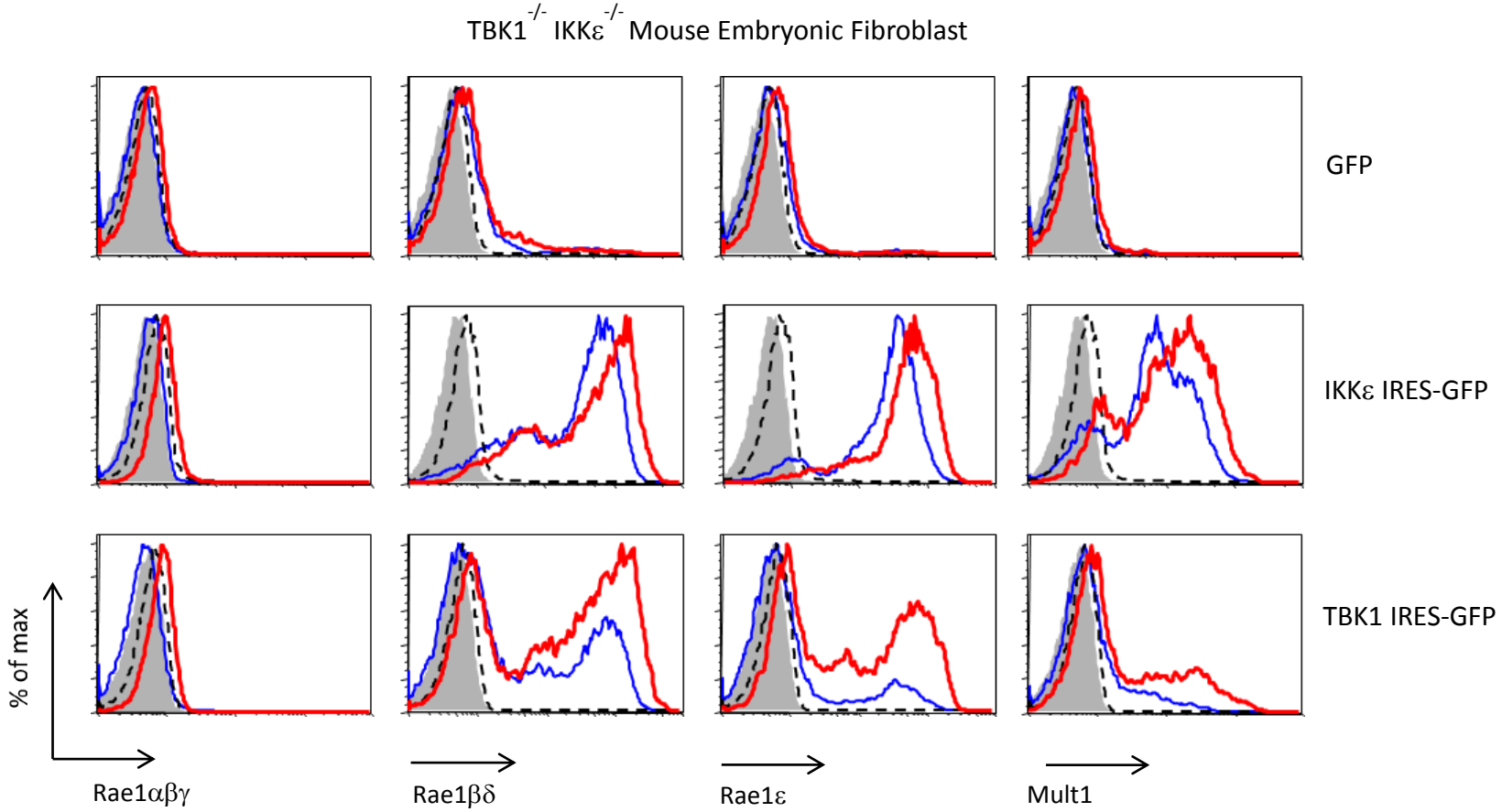

Fig. 3.11 Reconstitution of TBK1$^{-/-}$ IKKε$^{-/-}$ MEF cells with TBK1 or IKKε. TBK1$^{-/-}$ IKKε$^{-/-}$ MEF cells were transduced with MSCV IRES-GFP vectors encoding TBK1 or IKKε or the empty MSCV IRES-GFP vector and FACS-sorted for GFP expression. The sorted cells were cultured for 8 days and treated with DMSO (blue line) or 10 μM Ara-C (red line) for 18 hours. Isotype stainings of DMSO-treated (filled histograms) or Ara-C-treated (dashed lines) cells are shown.

3.4 DNA damage-mediated phosphorylation of IRF3 and TBK1 is dependent on ATR

3.4.1 TBK1 phosphorylation in response to Ara-C depends on ATR

As the initiation of DNA damage signaling by ATM/ATR is required for the upregulation of NKG2D ligands (Gasser et al, 2005), it was of interest if ATM/ATR is required for the phosphorylation of TBK1. We treated BC2 and Yac-1 cells with an inhibitor against both ATM and ATR or with a specific ATM inhibitor. After pre-treatment with the ATM/ATR inhibitor, TBK1 phosphorylation on Ser-172 was diminished in Ara-C-treated BC2 and untreated Yac-1 cells, but was maintained in cells treated with an ATM-specific inhibitor (Fig. 3.12). As an ATR-specific inhibitor was not available, we inferred through this observation that ATR, as opposed to ATM, has a bigger role in TBK1 activation.

3.4.2 IRF3 phosphorylation in response to Ara-C depends on ATR

As IRF3 is a target gene of TBK1, it was likely that the phosphorylation of IRF3 will require the activation of ATR and if so, it would further underline the role of ATR in the TBK1/IRF3 pathway. Indeed, similar to the ATR-dependent phosphorylation of TBK1 (S172), the phosphorylation of IRF3 on Ser-388 was reduced when the BC2 cells were pretreated with the ATM/ATR inhibitor (CGK 733) before DNA damage induction (Fig. 3.13). In accordance to the role of TBK1 in the constitutive expression of Rae1 in Yac-1, the levels of IRF3 phosphorylation were reduced when Yac-1 cells were treated with CGK 733 for 18 hours (Fig. 3.13). Interestingly, unlike for TBK1, the phosphorylation of IRF3 (S388) was reduced in BC2 cells when they were pretreated with the ATM-specific inhibitor (Fig. 3.13). However, the phosphorylation of IRF3 (S388) in Yac-1 cells remained unchanged.

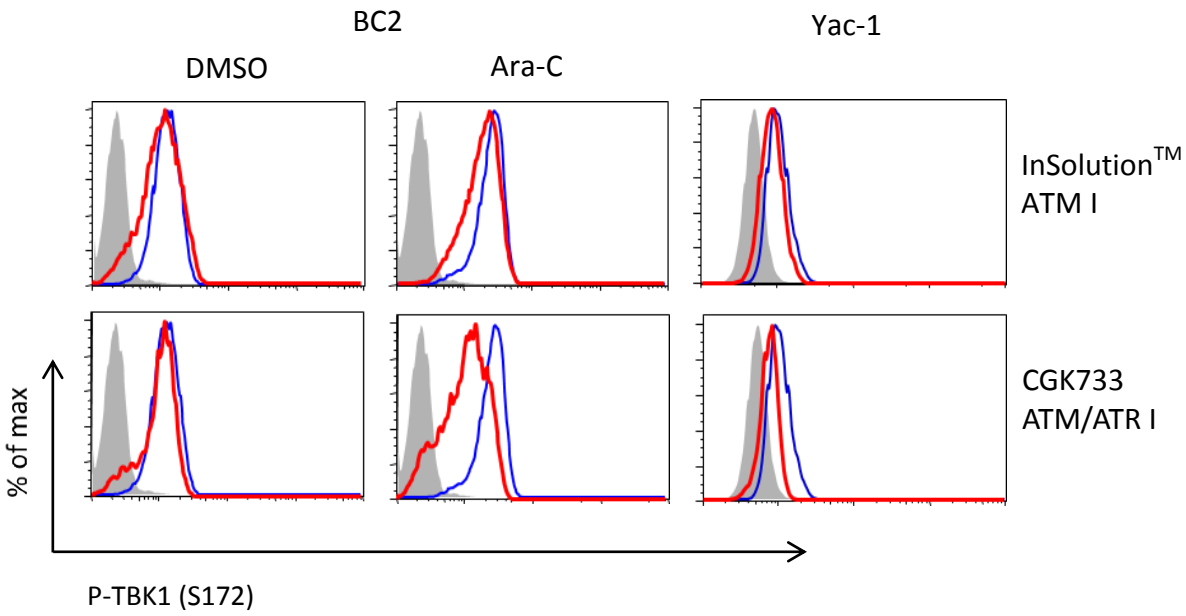

Fig. 3.12 TBK1 phosphorylation is reduced during ATR inhibition. BC2 cells pretreated with 10 μM of InSolution™ ATM-specific inhibitor or 10 μM of CGK733 (which inhibits both ATM and ATR) (red line) or DMSO (blue line) for 1 h, followed by treatment with DMSO or 10 μM Ara-C for 18 hours before they were stained for phospho-TBK1 (S172). Yac-1 cells treated with 10 μM of InSolution™ ATM-specific inhibitor or 10 μM of CGK733 (red line) or DMSO (blue line) for 18 hours were stained.

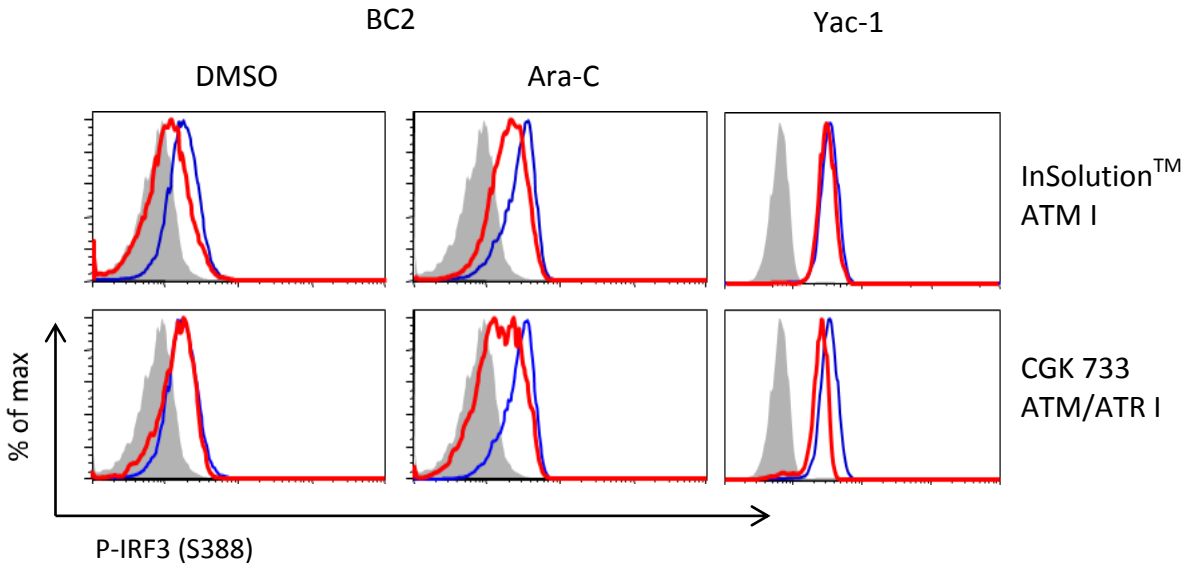

Fig. 3.13 IRF3 phosphorylation is reduced during ATR inhibition. BC2 cells pretreated with 10 μM of InSolution™ ATM-specific inhibitor or 10 μM of CGK733 (which inhibits both ATM and ATR) (red line) or DMSO (blue line) for 1 h, followed by treatment with DMSO or 10 μM Ara-C for 18 hours before they were stained for phospho-IRF3 (S388). Yac-1 cells treated with 10 μM of InSolution™ ATM-specific inhibitor or 10 μM of CGK733 (red line) or DMSO (blue line) for 18 hours were stained.

3.5 ATM/ATR interacts with SIKE during DNA damage response

3.5.1 Potential ATM/ATR substrate sites in Sike1

ATM kinase family members (including ATR and related kinases) phosphorylate substrates with consensus Ser-Gln (SQ) and Thr-Gln (TQ) motifs that are located immediately after negatively-charged and hydrophobic amino acids (Kim et al, 1999, O'Neill et al, 2000). In a large-scale proteomics study conducted by Matsuoka et al, over 700 proteins were identified to be ATM/ATR substrates in response to DNA damage (Matsuoka et al, 2007). Analysis of the protein list indicated that SIKE1 is an ATM/ATR target. In accordance, analysis of the human and mouse SIKE1 sequences revealed a potential ATM/ATR phosphorylation motif (TQ/SQ) (Fig. 3.14).

3.5.2 Co-localization of phosphorylated ATM and Sike1

To determine if ATM/ATR can phosphorylate Sike1, BC2 and Yac-1 cells were co-stained with antibodies against murine Sike1 and phospho-ATM (Ser-1981). The phosphorylation of ATM at Ser-1981 correlates with its activation and kinase activity in response to DNA damage (Bakkenist and Kastan, 2003; Lee and Paull, 2005). Sike1 and activation-specific phospho-ATM (Ser1981) are co-localized in the cytoplasm of Yac-1 cells (Fig. 3.15A), suggesting that cytoplasmic ATM may interact with and phosphorylate Sike1. Although ATM is thought to be mainly a nuclear protein, it was recently shown that ATM can translocate to the cytoplasm in response to DNA damage (Wu et al, 2006; Hinz et al, 2010). The co-localization of Sike1 and phospho-ATM (S1981) was also observed in some untreated BC2 cells (Fig. 3.15B), indicative of the low levels of ATM activity even when Rae1 is not expressed. As a potential target of the ATM/ATR-mediated DNA damage signaling pathway as well

as an inhibitor of a major mediator of innate immune signaling, Sike1 could link the TBK1/IKKε signaling pathway with the DDR for NKG2D ligand regulation.

Sike1

```
        10          20          30          40          50          60
MSCTIEKILT DAKTLLERLR EHDAAAESLV DQSAALHRRV AAMREAGAVL PEQYQEDASD
        70          80          90         100         110         120
VKDMSKYKPH ILLSQENTQI RDLQQENREL WVSLEEHQDA LELIMSKYRK QMLQLMVAKK
       130         140         150         160         170         180
AVDAEPVLKA HQSHSAEIES QIDRICEMGA VMRRAVQVDD NQFCKVQERL AQLELENKEL
       190         200
RELLSISSES LQVGKESSVA PASQTIK
```

Fig. 3.14 Potential ATM/ATR phosphorylation site in Sike1. Consensus ATM/ATR recognition sites have a phospho-Serine or phospho-Threonine site followed by a Glutamine (SQ/TQ) and preceded by Leucine (L) or similar hydrophobic amino acids at positions -1 and -3 from the phosphorylated amino acid. The Serine at position 74 (green highlight) in Sike1 fulfills the requirement of a potential ATM/ATR recognition motif (red bracket).

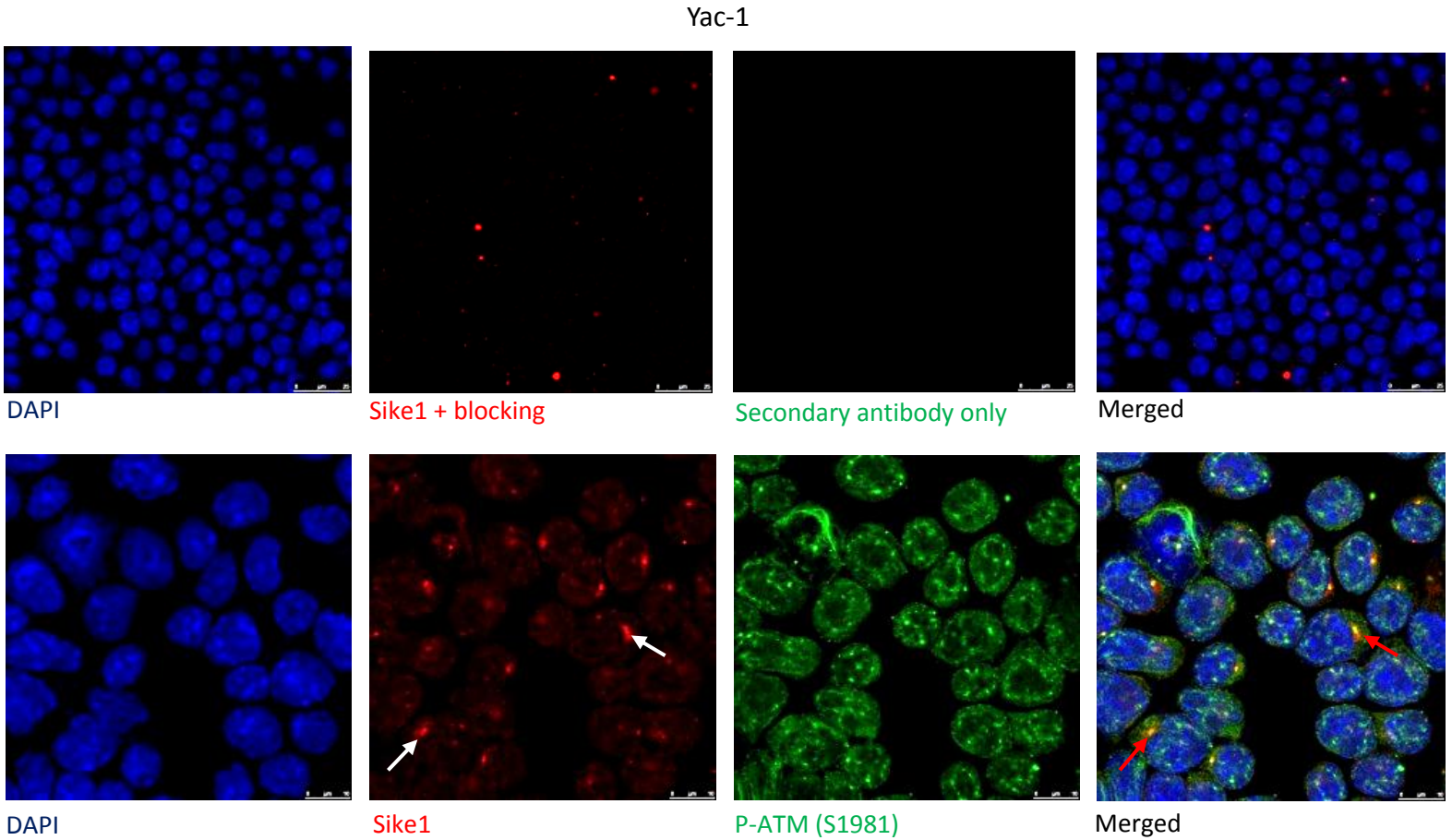

Fig. 3.15 (A) Co-localization of Sike1 with P-ATM (S1981) in Yac-1 cells. Yac-1 cells were fixed and stained with Sike1-specific (red) and activation specific P-ATM (S1981) (green) antibodies. DAPI is specific for nuclei staining. Sike1 is localized in the cytoplasm (white arrows) and co-localized with cytoplasmic P-ATM (red arrows). As a control, Yac-1 cells were stained with Sike1 antibody pre-incubated with Sike1 blocking peptide and secondary antibodies.

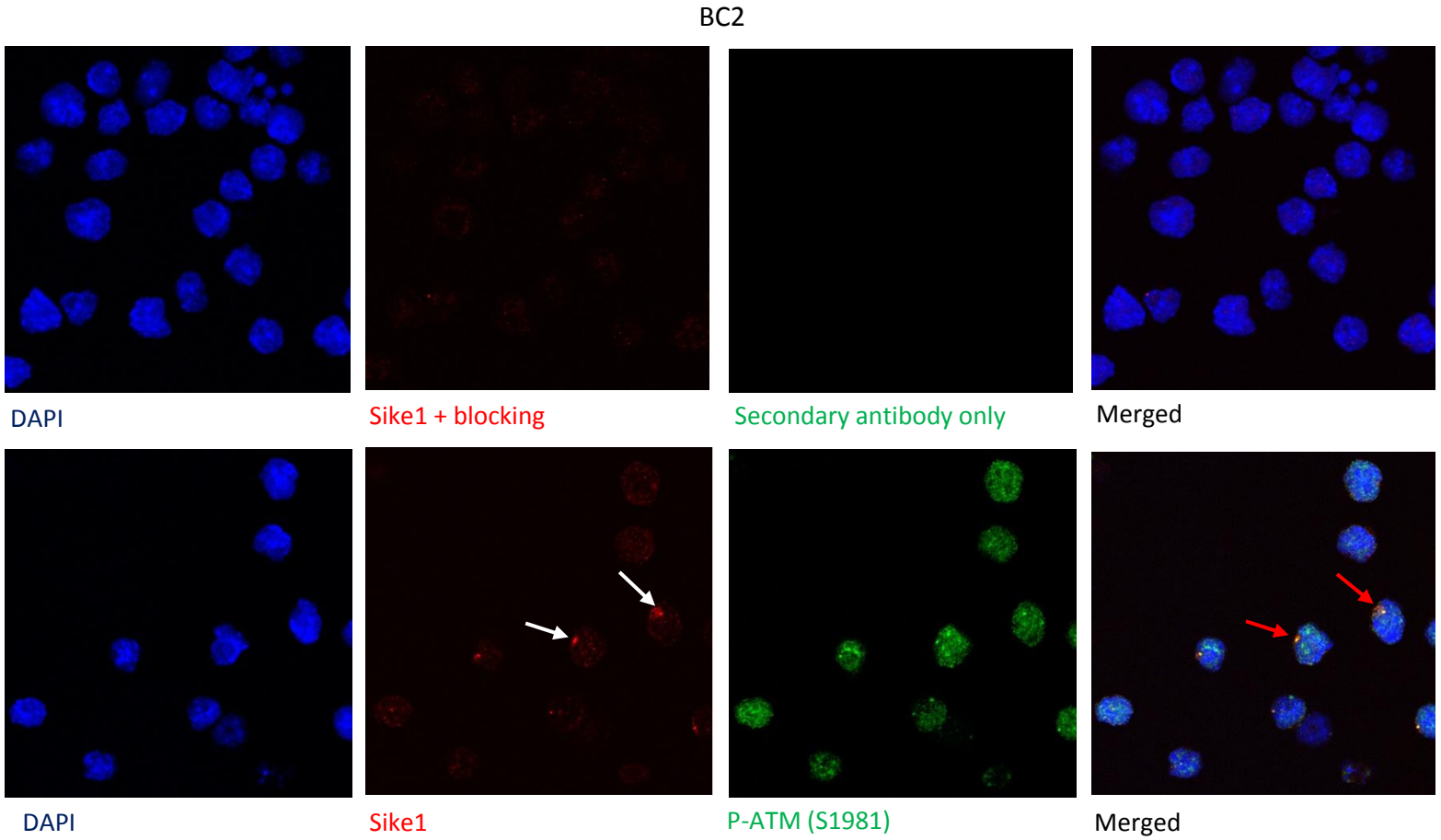

Fig. 3.15 (B) Co-localization of Sike1 with P-ATM (S1981) in BC2 cells. BC2 cells were fixed and stained with Sike1-specific (red) and activation specific P-ATM (S1981) (green) antibodies. DAPI is specific for nuclei staining. Sike1 (white arrows) is co-localized with P-ATM (red arrows). As a control, BC2 cells were stained with Sike1 antibody pre-incubated with Sike1 blocking peptide and secondary antibodies.

CHAPTER 4: DISCUSSION

The induction of NKG2D ligands through genotoxic stress allows the innate immune system to distinguish tumorigenic cells from normal cells (Gasser et al, 2005). Although ATM/ATR was previously shown to play a role for the induction of NKG2D ligands, the pathway linking ATM/ATR to the ligands remained to be elucidated. Our results have provided evidence that the link between the DNA damage response and these ligands lies in the TBK1/IRF3 pathway.

The nuclear translocation of IRF3 in response to DNA damaging agents was observed in a study conducted by Kim et al (1999). Our own experiments in our cell systems confirmed that the expression of Rae1 correlates with the activation status of IRF3. Translocation of IRF3 into the nuclei of Yac-1 cells and BC2 cells treated with Ara-C further supported the activation of IRF3 during DNA damage. Although IRF3 was found to be involved in the expression of Rae1, much remains to be determined concerning IRF3 target genes induced during DDR. Of interest was that the DDR led to lower production of IFN-β than treatment with Poly I:C, which likely suggests that DNA damage failed to activate IRF7, which is downstream of IFN-α/β and required for enhancement of type 1 IFN expression (Honda and Taniguchi, 2006). In line with our observation that the direct addition of type 1 IFN failed to induce the expression of NKG2D ligands on BC2 cells, it is likely that Rae1 is regulated directly by IRF3 and not downstream of the type 1 IFN receptor. The presence of consensus IRF-binding sequences on the Rae1 promoter gives plausibility to this notion. The production of IFN-β during DNA damage, however, seems to suggest that the interferon response is triggered in parallel with NKG2D ligands. The type 1 IFN induction by the DDR will reconcile with the observation by Takaoka et al (2003) that cancer cells were more susceptible to apoptosis when treated with type 1 IFNs, making it the other anti-oncogenic arm of the DDR. Interestingly, the DDR resulted in

a greater IP-10 gene activation. As IP-10 is known to be induced by TNF-α (Ohmori and Hamilton, 1995), it is possible that TNF-α is produced during the DDR and that DDR-induced TNF-α may augment the production of IP-10 in response to DNA damage. However, as neither IP-10 nor TNF-α was able to induce Rae1 expression when added to non-tumour cells (data not shown), we speculate that although IP-10 is induced during DNA damage, it has no direct effect on Rae1 expression.

Our subsequent experiments utilizing knockdowns or pharmacological inhibition of TBK1 and IRF3 and then phospho-flow staining of both implicated the TBK1/IRF3 pathway in mediating ligand induction in both genotoxic drug-induced upregulation of ligands and the maintenance of their constitutive expression. The use of flow cytometry to quantify levels of phosphorylation on specific proteins is new and technically challenging. The inconsistencies observed in the basal phosphorylation of TBK1 in DMSO-treated BC2 cells (Fig. 3.6 and Fig. 3.12) could be a result of technical variations or inevitable mutation of the cells in prolonged culture. Reconstituting TBK1 into TBK1-deficient MEF cells was sufficient to induce the expression of Rae1. This spontaneous upregulation of Rae1 in the non-tumour MEF cells can be speculated to have been caused by hyperactivation of the pathway as a result of unregulated expression of TBK1 or IKKε. The TBK1/IRF3 pathway orchestrates many important pro-inflammatory and antiviral responses. It is in turn initiated by many pattern recognition receptors e.g. toll-like receptors and cytoplasmic DNA/RNA sensors e.g. RIG-1/MDA5 (Palm and Medzhitov, 2009; Takeuchi and Akira, 2010). Any possible convergence of these other innate immune pathways with the anti-oncogenic DDR pathway would be interesting to explore.

From our ATM/ATR inhibition experiments, we postulate that ATR is required to phosphorylate and fully activate TBK1 during genotoxic stress. The possibility of whether ATR can directly phosphorylate Ser172 of TBK1, which is essential for activation, remains unclear. However neither TBK1 nor IRF3 was identified as phosphorylated targets during DNA damage in the large-scale proteomic screen conducted by Matsuoka et al (2007). It is possible that this pathway is cell type-specific, as the authors have used transformed human embryonic HEK293T cells, whereas we have used cells derived from B cell and T cell lymphomas. Another possibility is that ATR-dependent activation of TBK1 is indirect and requires intermediate molecules.

While we observed that ATM was required for the phosphorylation of IRF3 in BC2 cells (Fig. 3.13), the S172 phosphorylation of the upstream kinase TBK1 did not seem to depend on ATM (Fig. 3.12). This allows us to postulate that ATM may be regulating the pathway indirectly through lifting of the inhibitory effect of Sike1 on TBK1. The fact that Sike1 was identified as a phosphorylation target in the proteomic screen and our observation of co-localization between Sike1 and ATM supports this possibility (Fig. 3.15B). The phosphorylation of ATM and its co-localization with Sike1 in the absence of Ara-C treatment hints that low levels of DNA damage are already present in steady-state BC2 cells. Thus we postulate that in BC2 cells, the pharmacological inhibition of ATM during DNA damage induction may cause Sike1 to remain associated with TBK1, and although the ATR-mediated S172 phosphorylation of TBK1 is not blocked by Sike1, it blocks the TBK1 kinase activity on IRF3. This remains possible as the predicted site of TBK1-IRF3 interaction was reported to be at the C-terminus of TBK1 and away from S172 (Huang et al, 2005). In addition to the microscopy, co-immunoprecipitation can be performed to determine

interactions between ATM/ATR, Sike1 and TBK1 in future studies. The predicted ATM/ATR phosphorylation site on Sike1 can also be mutated to demonstrate the requirement of ATM/ATR in its phosphorylation (Fig. 3.14). On the other hand, in Yac-1 cells, ATM does not seem to have a role in regulating the constitutive phosphorylation of both TBK1 and IRF3. This observation corroborates with the findings made by Gasser et al (2005) that ATR had a predominant role in tumour cells expressing NKG2D ligands constitutively while ATM was involved in the drug-mediated upregulation of NKG2D ligands in non-cancerous fibroblasts (Gasser et al, 2005).

The spectral limitations of the fluorophores coupled to the secondary antibodies we used in confocal microscopy prevented us from studying the co-localization of ATM, Sike1 and TBK1 concurrently and the lack of a suitable commercially-available anti-phospho-ATR antibody for immunocytochemistry use has also hampered the investigation into whether ATR can co-localize with Sike1. Nevertheless the abrogation of Rae1 upregulation during DNA damage when Sike1 was overexpressed strongly hints at its role in the pathway. While it is clear that the activation of TBK1 and IRF3 is required for the induction of NKG2D ligands in both BC2 and Yac-1 cells, the differential requirement of ATM for the activation of the TBK1-IRF3 pathway hints at the cell type-specific regulation of the pathway. The general model of DDR-induced mechanisms underlying the expression of Rae1 is hence summarized in figure 4.1.

In addition to cell type-specificity, the nature of the DNA damage may activate NKG2D ligands differentially. Questions regarding whether a specific type of DNA lesion is required for Rae1 induction would be interesting to explore. The detection of foreign cytoplasmic DNA/RNA due to pathogen invasion or the presence of self DNA

in the cytoplasm caused by DNA damaging agents may plausibly lead to the upregulation of Rae1 expression. These possibilities offer exciting areas of research into the mechanisms of NKG2D ligand regulation.

In summary our data suggest that DNA damaging agents and tumorigenesis lead to the activation of the TBK1/IRF3 pathway, possibly through the involvement of both ATM and ATR. ATR activation culminates in TBK1 activation, while ATM may possibly be required to allow TBK1 and IRF3 to interact. The activation of the TBK1/IRF3 pathway is required for the expression of Rae1 on tumour cells, possibly through direct binding of IRF3 to the Rae1 promoter. Rae1 displayed on nascent tumour cells can lead to the activation of innate immune cells such as NK cells or enhance the responses of certain subset of T cells to induce cytolysis. We have proposed the mechanistic link between the well-defined immune recognition pathway and the genotoxic stress response.

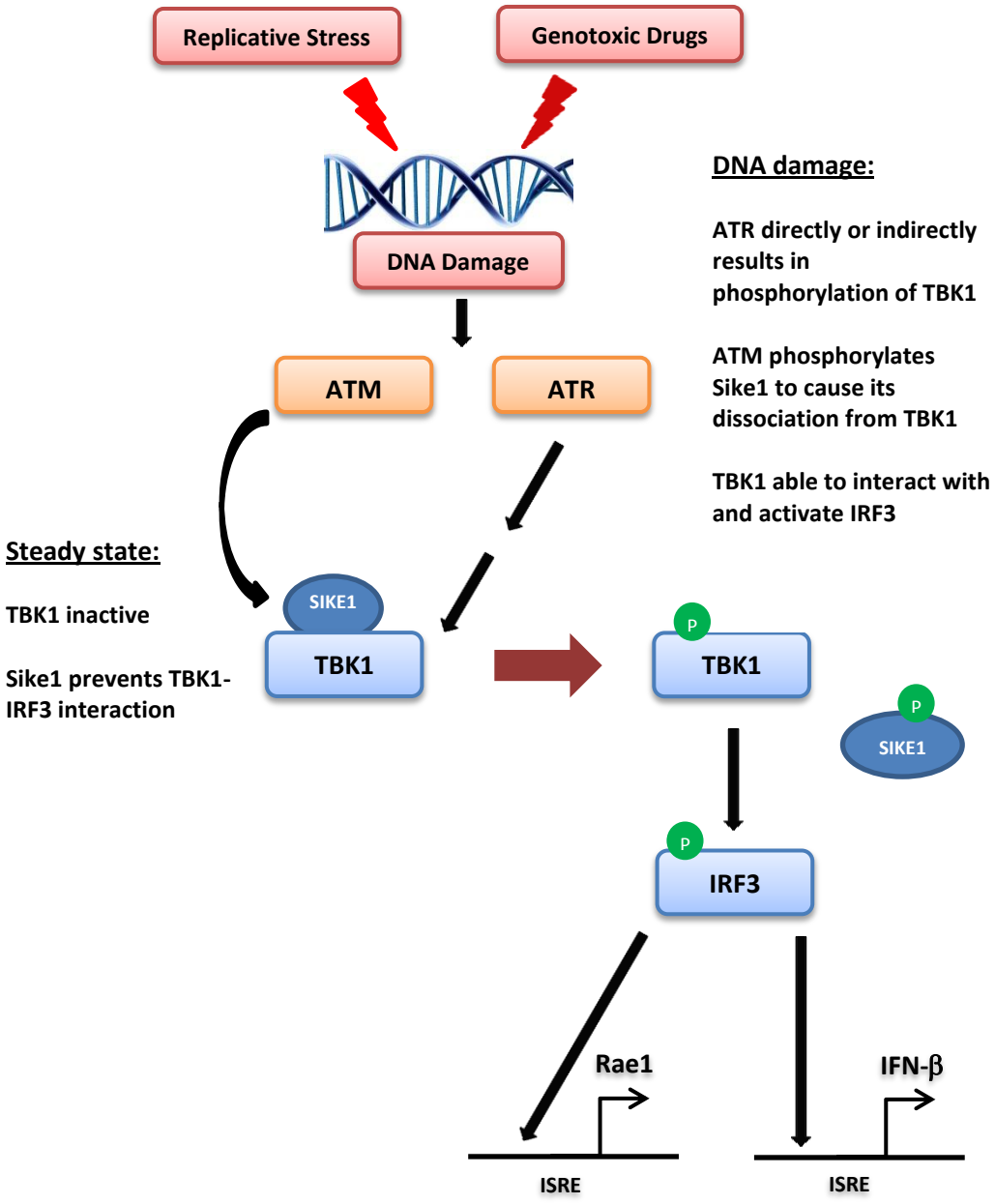

Fig. 4.1 Proposed model of DNA damage leading to Rae1 induction. Upon sensing of DNA damage, ATM phosphorylates Sike1 and concurrently, ATR activates TBK1. The inhibition of Sike1 on TBK1 is lifted activated TBK1 can in turn activate downstream IRF3. IRF3 translocates to the nucleus and drives the expression of Rae1. IRF3 also drives the production of type 1 IFN in parallel.

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