The Role of STING in Cancer

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A THEIS SUBMITTED

FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

NATIONAL UNIVERSITY OF SINGAPORE

2015

DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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29 April 2015

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Acknowledgements

I would like to thank all who have helped me during my project. Firstly, I would like to express my sincere gratitude to my supervisor Dr. Stephan Gasser for his generous guidance and support throughout my research and thesis writing. Dr. Gasser's expertise and devotion to science will always inspire me.

I would also like to thank Prof. Lina Lim and Dr. Alessandra Mortellaro for their time to provide me with wise advice and feedback on my project. Special thanks to Dr. Yujia Shen and Dr. Nina Le Bert for giving me invaluable advice and assistance at the start of the project. I would also like to express my gratitude to Dr. Paul Hutchinson and Guohui Teo for providing technical assistance of the flow cytometer and cell sorter. I would like to thank all past and present lab members for the generous help, the friendships and encouragements they had provided. Finally, I would like to express my sincere thanks to my family and friends who had always supported me.

Summary

Innate immunity is the first line of host defense against infection by microbial pathogens, including viruses, bacteria and parasites. Recognition of nucleic acids by innate immune sensors is an important means to detect and protect against of pathogens in infected cells. In this project, we show that the cytosolic DNA sensor STING may regulate the levels of cytosolic DNA in cancer cells. Activation of STING in infected cells leads to the production of type I interferons. In accordance, constitutive expression of type I interferons partially depended on STING in cancer cells. In summary, our data suggest that STING contributes to recognition of cytosolic DNA presence in cancer cells.

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List of Abbreviations

A

AIM2: absent in melanoma 2

Ara-C: Arabinosylcytosine

ATM: ataxia telangiectasia, mutated

ATR: ATM- and Rad3-related

B

BASC: BRCA1-associated genome surveillance complex

BRCA1: breast cancer type 1 susceptibility protein

C

CDN: cyclic dinucleotide

cGAMP : cyclic GMP-AMP

cGAS: cyclic guanosine monophosphate-adenosine monophosphate synthase

CHK: checkpoint kinase

CRISPR: Clustered regularly interspaced short palindromic repeats

D

DAMP: damage-associated molecular pattern

DAPI: 4',6-diamidino-2-phenylindole

DDR: DNA damage response

DDX41: DEAD(Asp-Glu-Ala-Asp) box polypeptide 41

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DNA-PK: DNA protein kinase

DNase: deoxyribonuclease

DSB: double strand break

dsDNA: double-stranded DNA

E

ER: endoplasmic reticulum

F

FBS: fetal bovine serum

FGF2: fibroblast growth factor 2

G

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GFP: green fluorescent protein

H

HDAC: histone deacetylases

HEPES: 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

HPRT: Hypoxanthine-guanine phosphoribosyltransferase

HSV1: herpes simplex virus 1

I

IFI16: interferon gamma inducible protein 16

IFN: interferon

IRF3: IFN-regulator factor 3

ISGF3: IFN-stimulated gene factor 3

ISRE: IFN-stimulated response elements

J

JAK1: Janus kinase 1

JNK: Jun N-terminal protein kinase

M

MDR: multidrug resistance

MNDA: myeloid cell nuclear differentiation antigen

MRP: MITA-related protein

N

NF-κB: nuclear factor-κB

NHEJ: non-homologous end joining

HDR: homology-directed repair

NK: Nature killer

NLR: NOD-like receptor

NOD: nucleotide-binding and oligomerization domain

P

PAMP: pathogen-associated molecular pattern

PI3K: phosphoinositide 3-kinase

PIKK: PI3K-related protein kinase

PRR: pattern recognition receptor

R

RGS5: regulator of G-protein signaling 5

RIG-I: retinoid acid-inducible gene-I

RIPA: Radio-Immunoprecipitation Assay

RLR: RIG-like receptor

RNA: Ribonucleic acid

RNase: ribonuclease

S

SAPK: stress-activated protein kinase

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

ssDNA: single-stranded DNA

STAT: signal transducer and activator of transcription

STING: stimulator of interferon gene

T

TBK1: TANK-binding kinase 1

TLR: Toll-like receptor

TYK2: tyrosine kinase 2

V

VEGF: vascular endothelial growth factor

Z

ZBP1: Z-DNA-binding protein 1

Chapter 1

Introduction

In addition to the cell intrinsic barriers to tumorigenesis and tumor formation mediated by tumor suppressors such as p53, the immune system has also been involved in tumor surveillance in several aspects (Lesokhin et al., 2015). The innate immune response consists of a series of cellular sensors and signaling pathways that activates defense mechanisms in response to microbe invasion (Czarny et al., 2015). The innate immune system responds to the presence of pathogens or danger by recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular pattern (DAMPs) respectively, leading to altered gene expression, immune effector mechanisms and ultimately a return to homeostasis. PAMPs and DAMPs are detected by several classes of host pattern recognition receptors (PRRs) including the Toll-like receptors (TLRs), RIG-like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptors. These innate immunity responses finally result in the production of type I interferon (IFN) (Shimizu et al., 2014).

1.1 DNA Damage Response (DDR)

1.1.1 Overview of the DNA damage

Accumulation of DNA damage has been involved in the phenotypic manifestations of aging in rodents and humans (Lombard et al., 2005). DNA damage can be induced by variety of activities such as endogenous or exogenous stresses, including oxidative stress, telomere erosion, oncogenic mutations, genotoxic stress and metabolic stress (Lo ́pez-Otı´n et al., 2013). The presence of DNA damage can cause cell-cycle arrest, apoptosis and cell

senescence. Accumulation of senescent cells can impair tissue regeneration and homeostasis which leads to metabolic dysfunction. In addition, it is reported that chronic inflammation associated with senescence has a crucial role in the processing of age-related diseases such as diabetes and cancer.

1.1.2 Overview of the DNA damage response

To defend alterations to the nuclear DNA, all organisms have developed a complex and efficient system for repairing DNA damage and eliminating cells that cannot be repaired, called DNA damage response (DDR) (Ciccia and Elledge, 2010). DDR is a signal-transduction pathway that directs and regulates cell-cycle transitions, DNA replications, DNA repair and apoptosis. The major regulators of DDR are the phosphoinositide 3-kinase (PI3K)-related protein kinases (PIKKs), including ataxia-telangiectasia mutated (ATM) and ATM and RAD3-related (ATR). ATM and ATR have similar biochemical and functional characteristics (Piotr Czarny, 2015). Both of them are large kinases with important sequence homology and tendency to phosphorylate Ser or Thr residues which are followed by Gln (Weber, 2014). Moreover, both phosphorylate a common set of substrates that leads to cell-cycle arrest and DNA repair. The ATM and ATR kinase are activated by different genomic insults. ATM seems to be activated primarily by double strand breaks (DSBs), while ATR is mainly involved in the response to stalled replication forks, although it can participate in the DDR to DSBs.

Upon DNA damage, ATM and ATR phosphorylate a multitude of substrates to induce the required cellular response (Ciccia and Elledge, 2010). Two classes of proteins are cooperated with them to transduce the DNA damage signal: transducer kinases and checkpoint mediators. Checkpoint mediators, such as MDC1, 53BP1 and BRCA1 for ATM (Shiloh and Ziv, 2013) and TopBP1 and claspin for ATR (Liu, 2006), can contribute to the activation of ATM and ATR by binding to the lesions and facilitating recruitment of DDR factor to the damage sites indirectly (Canman, 2003; Marechal, 2013). Transducer kinases are involved in spreading of the DNA damage signal through a phosphorylation way. Checkpoint kinase 1(CHK1) for ATR and CHK2 for ATM is known as the most important transducer kinases (Bekker-Jensen and Mailand, 2010).

ATM creates a homodimer in its inactive form, which is recruited to the site of DSB by the Mre11-Rad50-Nbs1 (MRN) complex, further undergoes auto-phosphorylation and separates into two active monomers (Bakkenist, 2003). After recruitment and activation, ATM and ATR interact with many mediators and performing proteins, including CHK1 and CHK2 involved in the cell cycle control, p53, which is a multifunctional protein, is crucial for cell survival and a famous tumor suppressor, breast cancer type 1 susceptibility protein (BRCA1)-associated genome surveillance complex (BASC) involving DNA damage repair proteins (Wang, 2000; Kim,1999), histone deacetylases 1 and 2 (HDAC1 and HDAC2) responsible for

remodeling chromatin structure, and transcription factor FOXO3, regulating genes involved in DNA repair (Schmidt et al.,1999;Tran et al., 2002; Thurn,2013).

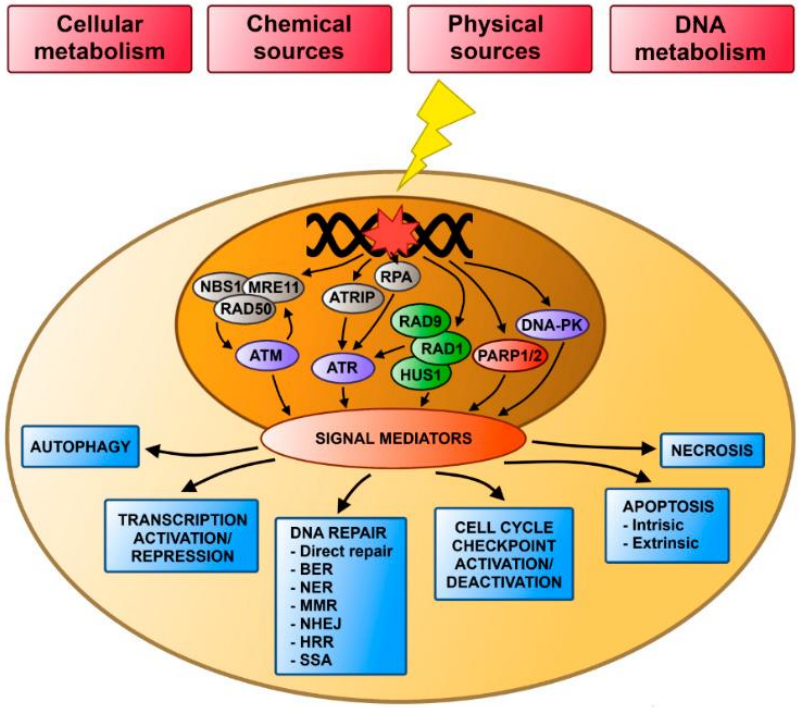

Figure 1.1 Overview of DNA damage responses

DNA damage can be induced by both exogenous chemical and physical factors, and by endogenous influences following from cellular and DNA metabolism. The DNA damage induction triggers the DNA damage response (DDR). Three proteins from the phosphatidylinositol 3-kinase-like protein kinases (PIKKs) family plays a major role in DDR: ataxia telangiectasia mutated (ATM), DNA protein kinase (DNA-PK) and ataxia telangiectasia and Rad3 related (ATR), two proteins of the poly (ADP-ribose) polymerase (PARP) family: PARP1 and PARP2, and heterotrimeric complex of Rad9, Rad1 and Hus1 (9–1–1 complex). These proteins are activated by either DNA damage itself or by other proteins. After ATM, ATR, DNA-PK, PARP1/2 or 9–1–1 complexes are activated, they transfer signals via signal mediators to regulate many cellular processes, including DNA repair, cell checkpoint activation or deactivation, activation or silencing of transcription, apoptosis and autophagy. Image taken from Piotr Czarny et al., 2015.

1.1.3 The role of the DNA damage response in tumorigenesis

Genomic instability especially DNA damage is one of the hallmarks of cancer (Stratton et al., 2009). Although the specific DDR flaws remain unclear in most cancers, there is an incontrovertible connection between a particular DDR defect and the neoplastic phenotype in several cases. For instance, around 15% of sporadic colorectal tumors show abnormal changing in length of dinucleotide repeat sequences, which is a characteristic of microsatellite instability, which possibly be caused by defective mismatch repair that results in an inability to repair DNA replication errors. Microsatellite instability is also found in the familial form of the disease such as hereditary non-polyposis colorectal cancer (HNPCC), which is associated with loss of function mutated mismatch repair genes, such as MSH2 and MLH1 (Renwick et al., 2006). ATM mutations increase the susceptibility of carriers to cancer and were found in 0.5-1.0% of the population approximately (Swift et al., 1987). Mutations in ATR are unusual and probably only result in viability when they are heterozygous.

Moreover, DDR has been reported to provide an important barrier to tumorigenesis (Bartek et al., 2001, Bartkova et al., 2005; Gorgoulis et al., 2005). Uncontrolled cell proliferation, which is induced by oncogene activation or inactivation of certain tumor suppressors, leads to DNA-replication stress and continuous DNA-damage. All these events will lead to activation of the ATM/ATR-mediated signaling. The DDR is also

activated in early neoplastic lesions and exerts as a barrier against malignant tumor (Bartkova et al., 2005; Gorgoulis et al., 2005). Inactivation of DDR, which is arising through mutational or epigenetic inactivation of components in DDR, is further selected for during tumor development, which could facilitate tumorigenesis (Halazonetis et al., 2008). The DDR model explains the high frequency of DDR dysfunction or defects in different human cancers.

1.2 Type I Interferons

1.2.1 Overview of interferons (IFNs)

Interferons (IFNs) are important immune-modulatory cytokines that are induced in response to a variety of viral and bacterial infections and help establish anti-microbial innate immunity (Peskta et al., 2004). According to their structure and utilization of specific receptors, IFNs are classified into 3 distinct types: type I IFNs, type II IFNs, and type III IFNs. Type I IFNs consist of 14 subtypes of IFNα, a single IFNβ, IFNκ, IFNω, IFNε, IFNδ and IFNτ. All type I IFN members share a common heterodimetric IFNα/β receptor that is composed of IFNAR1 and IFNAR2 (Pestka et al., 2004; Hertzog & Willianms, 2013). Type II IFNs are presented by a single member IFNγ, which interacts with a single IFNγ receptor with two chains, IFNGR1 and IFNGR2. Type III IFN is comprised of 3 members, IFN- λ1, IFN-λ2, and IFN-λ3 (also called IL-28A, IL-28B, and IL-29 respectively) that are closely related to the IL-10

family cytokines. Type I IFNs and the IFNα/β receptor, as well as the IFNγ receptor, are widely expressed in contrast to the restricted expression patterns of IFNγ, type III IFNs and the IFNγ receptor. IFNγ is mainly produced by T cells and NK cells and type III IFNs by leukocytes and epithelial cells (Pestka et al., 2004; Young et al., 2007; Kotenko et al., 2011). Type III IFNs are suggested to protect epithelial tissue from viral and bacterial infection because of its primary expression by epithelial cells.

1.2.2 Type I IFN signaling pathway

The most well defined type I IFNs are IFNα and IFNβ (Hertzog & Willianms, 2013). Most types of cells produce IFNβ, while haematopoietic cells, especially plasmacytoid dendritic cells, are the predominant producers of IFNα. IFNβ is encoded IFNB gene, while 14 distinct genes encode IFNα various isoforms. Type I IFNs production is induced after the sensing of microbial products by pattern-recognition receptors (PRRs) (Paludan and Bowie, 2013; Goubau et al., 2013; Iwasaki, 2012) and by various cytokines.

In the canonical type I IFN-induced signaling pathway, IFNAR engagement was reported to activate the receptor-associated protein tyrosine kinase 2(TYK2) and tyrosine kinases Janus kinase 1 (JAK1), both of which could phosphorylate the potential cytoplasmic transcription factors signal transducer and activator of transcription 1 (STAT1) and STAT2 (Levy and Darnell, 2002; Stark and Darnell, 2012) (Figure1.2). Tyrosine phosphorylated STAT1 and 2

dimerize and translocate to the nucleus, where they bind with IFN-regulator factor 9 (IRF9) to form a trimolecular complex termed IFN-stimulated gene factor 3 (ISGF3). ISGF3 always binds to its cognate DNA sequences, which are known as IFN-stimulated response elements (ISREs). The consensus sequence is TTTCNNTTTTC. Further they directly activate the transcription of ISGs (MacMicking, 2012). On the contrary, most other cytokines activate STAT homodimers that bind to a distinct gamma-activated sequence (GAS; consensus sequence: TTCNNNGAA). Therefore, canonical type I IFN signaling induces a distinct subset of several hundred ISRE-driven ISGs, and most of them could build a cellular antiviral state (Schoggins et al., 2011; Rusinova et al., 2012).

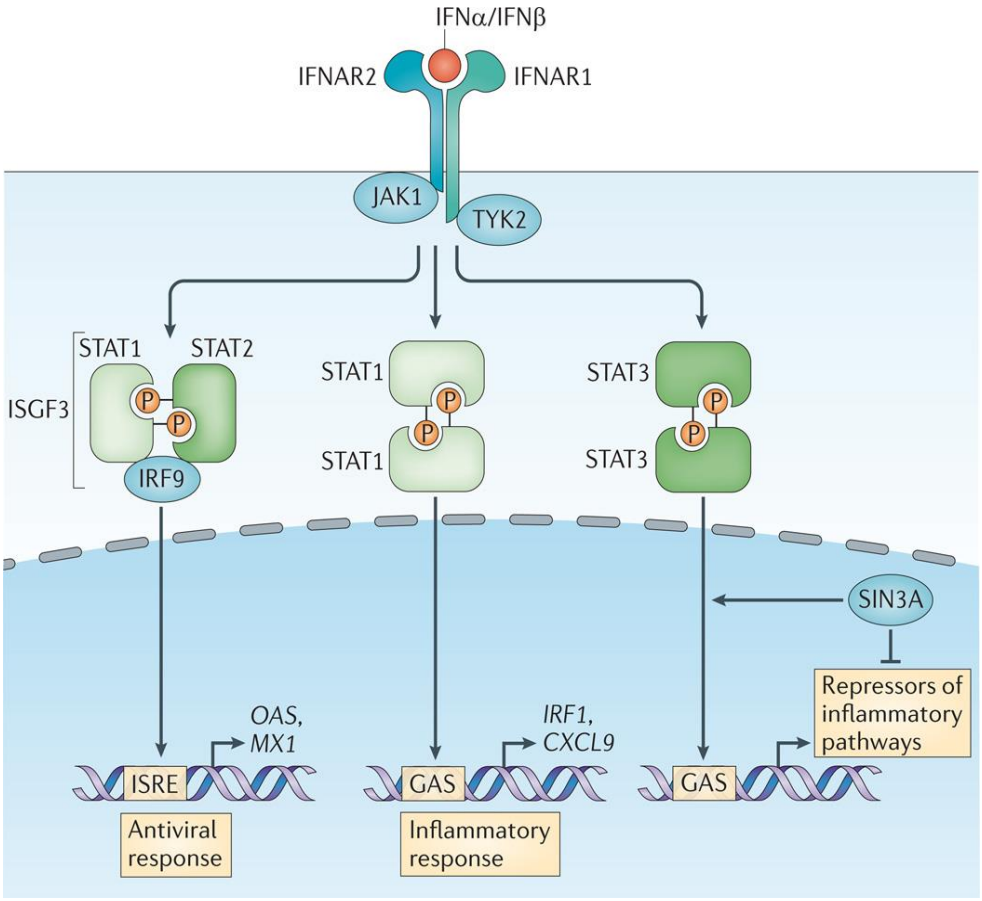

Figure 1.2 Overview of the IFN receptor signaling pathways
IFNAR is composed of IFNAR1 and IFNAR2 subunits, which activates JAK1 and TYK2 upon binding of IFNs. Subsequently, STAT proteins are recruited, which control distinct gene expression processes. ISGF complex binds to ISRE sequences to activate typical antiviral genes, while STAT1 homodimers bind to GASs to induce pro-inflammatory genes. STAT3 homodimers suppress pro-inflammatory gene expression indirectly by binding to co-repressor complex SIN3 transcription regulator homologue A (SIN3A). Image taken from Ivashkiv and Donlin, 2014.

1.2.3 Role of type I IFN in cancer

Type I IFN regulates more than 200 genes such as NF-κB, interferon regulatory factor 1 (IRF1), p53, STAT1 and interleukin enhancer binding factor 3 (NF-90) which control diverse cell processes such as growth (Petryshyn et al., 1984) and differentiation (Meurs et al., 1993) at a transcriptional level. Most of these effects contribute to the antiproliferative

activity of these cytokines by cell cycle arrest and/or apoptosis (Gil et al., 1998). IFNα and IFNβ possibly mediate antitumor activity through indirect mechanisms by regulation on immunomodulatory and anti-angiogenic responses. Type I IFNs can affect immune responses by the effects on myeloid (Luft et al., 1998), T and B cells (Le Bon et al., 2001), chemo-kinesis and chemotaxis, as well as promote the acquisition of cytotoxic activity of natural killer (NK) cells (Lee et al., 2000).

Another interesting antitumor mechanism of IFNs is the anti-angiogenic activity. These cytokines inhibit secretion of angiogenic factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2) that produced by tumor cells and regulator of G-protein signaling 5 (RGS5) that is a protein involved in angiogenic tumor vasculature (Zhu et al., 2008). IFNs have been proposed to be a potential therapy approach to overcome multidrug resistance (MDR). Multidrug resistance-associated proteins are overexpressed in several aggressive tumors such as pancreatic cancer (Vitale et al., 2007).

As a result of the anti-proliferative and differentiating effects of Type I IFNs, IFNα and IFNβ genes may possibly function as tumor suppressor. With previous study, there has been an increasing interest in a possible role of IFNα in combination with the chemo-radio-immunotherapy in the treatment for pancreatic cancer, as shown by several phase II and III trials (Neoptolemos et al., 2004). All these studies reported a potential antitumor activity using an

IFNα-based chemo/radiation regimen in cancer, but the side effects are still unclear. On the contrary, with the detailed study of antitumor effects of IFNα, those of IFNβ are not well established. However, IFNβ is significantly more effective in vitro than IFNα in inducing cell growth inhibition in both exocrine and endocrine tumors of the pancreas and adrenal cancer (Vitale et al., 2006; Koetsveld et al., 2006). In these tumors, IFNβ is more potent in inducing both apoptosis and cell cycle arrest in late S-phase than IFNα. IFNβ has a higher binding affinity than IFNα and has higher synergistic interaction on tumor cell growth inhibition when combined with other antitumor agents (Croze et al., 1996).

1.3 Nucleic acid sensing

The cellular innate immune system senses and eliminates microbes rapidly in a non-specific manner, which consists of different groups of cellular sensors (Wu and Chen, 2014). Sensors include the Toll-like receptors (TLRs), retinoid acid-inducible gene I (RIG-I)- like receptors (RLRs) and cytosolic nucleic acid sensors, in addition to nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs).

The innate immune response consists of a series of cellular sensors and signaling pathways that activates defense mechanisms in response to microbe invasion. The innate immune system responds to the presence of pathogens or

danger by recognition of pathogen-associated molecular patterns (PAMPs) or damage-assoicated molecular pattern (DAMPs) respectively, leading to altered gene expression, immune effector mechanisms and ultimately a return to homeostasis. PAMPs and DAMPs are detected by several classes of host pattern recognition receptors (PRRs) including the Toll-like receptors (TLRs), RIG-like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptors.

Many PRRs are able to trigger a TANK-binding kinase 1 (TBK1) and interferon regulator factors 3 (IRF3)-dependent type I IFN and cytokine response. Recently identified candidate cytosolic DNA sensors include Z-DNA-binding protein 1 (ZBP1); absent in melanoma 2 (AIM2); interferon gamma inducible protein 16 (IFI16); stimulator of interferon gene (STING); DEAD(Asp-Glu-Ala-Asp) box polypeptide 41 (DDX41) and cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS) (Sun et al., 2013; Takaoka et al., 2007; Yang et al., 2010; Yanai et al., 2009) IKKε is mainly expressed in lymphocytes, while TBK1 is constitutively expressed in embryonic and adult fibroblasts (Perry et al., 2004) and is required in these cells for normal IRF3 activation and IFN-beta production induced by dsRNA (Lam et al.,2014; Hemmi et al., 2004).

1.3.1 Cytosolic DNA sensors and tumorigenesis

IFI16 and AIM2, both of which are cytosolic DNA sensors of the PYHIN family, have been proposed to function as tumor suppressor. The PYHIN (pyrin and HIN200 domain-containing proteins) family consists of the mouse IFN-inducible genes Ifi200, p202a, p202b, p203, p204, myeloid cell nuclear differentiation antigen (Mnda1) and Aim2, as well as the human HIN-200 genes IFI16, MNDA, AIM2 and PYHIN1 (Gariglio et al., 2011). Many PYHIN family members were also reported to regulate proliferation, differentiation and transcriptional regulation (Asefa et al., 2004; Cresswell et al., 2005)

Overexpression of IFI16 protein in normal human diploid fibroblasts and prostate epithelial cells is a link to cellular senescence-associated permanent cell growth arrest (Choubey et al., 2008). According with this finding, expression of IFI16 was downregulated in breast cancer tissue (Fujiuchi et al., 2004) and transcriptional silencing of IFI16 by histone deacetylase was found in human prostate cancer cell lines (Alimirah et al., 2007). Increased expression of IFI16 in cells inhibits cell proliferation by promoting the p53/p21- and RB/E2F- mediated inhibition of cell-cycle progression, and downregulation of IFI16 could be responsible for oncogenesis (Liao et al., 2011; Ludlow et al., 2005). Furthermore, IFI16 is reported to have anti-tumoral activity by inducing apoptosis of tumor cells in vivo, through inhibiting neo-vascularisation, and increasing the recruitment of macrophages

(Mazibrada et al., 2010). AIM2 is often mutated in human melanomas, colorectal carcinomas, gastric and endometrial cancers (DeYound et al., 1997; Michel et al., 2010; Woerner et al., 2007). More studies indicate that overexpression of AIM2 suppresses proliferation and promotes the rate of apoptosis of several cells, and other members of HIN-200 family of proteins have the similar effect (Johnstone and Trapani, 1999; Landolfo et al., 1998). AIM2 expression inhibition in human diploid fibroblasts triggers the activation of DDR, which implicates a role of AIM2 in the maintenance of genomic stability in cells (Duan et al., 2011). Moreover, AIM2 is essential for IL-1beta induction by Epstein-Barr virus (EBV) genomic DNA and EBV-encoded small RNAs, which inhibits tumor growth and promotes survival rates by host responses respectively (Chen et al., 2012).

1.4 Stimulator of interferon genes (STING)

STING (stimulator of interferon genes) was identified by its ability to trigger innate immune gene transcription including the secretion of type I IFN in response to microbial invasion (Ishikawa and Barber, 2008). STING, which is also known as TMEM173, MTIA, ERIS and MPYS, is a 379 amino acid protein in human cells, comprising several transmembrane regions in its N-terminal region, which presents as a dimer in the endoplasmic reticulum (ER) (Barber, 2014). STING performs broad anti-pathogen functions, which is

independent of the TLR pathway and possibly be evolutionarily conserved, with homologs existing in Drosophila. For instance, after DNA virus, retrovirus or bacterial infection, STING-dependent signaling is activated when nuclear translocation of the transcription factors IRF3, nuclear factor-κB (NF-κB) and Jun N-terminal protein kinase/stress-activated protein kinase (JNK/SAPK) pathway happens, which facilitates the production of dozens of innate and adaptive immune modulatory proteins including pro-inflammatory cytokines such as CXCL10 and TNFα (Burdetter and Vance, 2013). According to the studies that have been conducted past few years, STING is considered to be effectively activated in the presence of cytosolic DNA that are generated from DNA viruses or bacteria such as herpes simplex virus 1 (HSV1) or Listeria (Sauer et al., 2011). Moreover, STING is able to be stimulated by transfected DNA including bacterial genomic DNA, purified viral DNA, plasmid DNA, calf thymus DNA, and synthetic double-stranded DNA (dsDNA) bound with cationic liposomes (Ishikawa et al., 2009). Such ligands cannot induce type I IFN secretion, as well as other innate immune proteins in *Sting*-deficient fibroblasts, conventional dendritic cell and macrophages, which suggests the core role of STING in innate immune system (Ishikawa et al., 2009).

Although it is reported that STING can directly bind to dsDNA species, its affinity is very low in mammalian cells (Civril et al., 2013). The search for DNA sensors that could facilitate STING activity led to the discovery of cyclic

GMP-AMP synthase (cGAS) that generates the cyclic dinucleotide (CDN), cyclic GMP-AMP (cGAMP) from GTP and ATP in a DNA-dependent manner (Sun et al., 2013; Burdette et al., 2011). In human cells, cGAS catalytic activity could be activated by sequence non-specific dsDNA greater than 45 base pairs. In mammalian cells, cGAS generated cGAMP binds to STING dimers on the ER to initiate innate immune gene production, but the mechanism is still unclear (Cai et al., 2014). In bacteria, cyclic di-AMP and cyclic di-GMP are secreted and function as ubiquitous second messengers to trigger a spectrum of responses to internal and external stimuli (Woodward et al., 2010). These CDNs can activate directly in the same way as cGAMP. Moreover, *cGAS*-deficient mice showed similar phenotypes to STING deficient mice in susceptibility to DNA pathogen infection and essentials for IFN production upon dsDNA treatment (Li et al., 2013). Activation of STING by DNA viruses induces trafficking from the ER area through the Golgi apparatus to perinuclear regions via a mechanism resembling non-canonical autophagy. This processing includes STING escorting TBK1 to endosomal or lysosomal regions, which likely harbor the transcription factors IRF3 and NF-κB (Ishikawa et al., 2009) (Figure 1.3).

Observing that activation of STING can induce potential innate immune transcriptional events has led to evaluation of whether ligands that activate STING signaling pathway could be useful as potent adjuvants in anti-pathogen and vaccine related strategies (Coban et al., 2013; Lemos et al., 2014). It is

worth to note that STING signaling has been shown to be important for the adjuvant effects of plasmid-based DNA vaccines (Ishikawa et al., 2009). For instance, mice lacking STING does not generate an adaptive immune responses to DNA vaccines. Hence, understanding the STING pathway possibly leads to the new generation of more effective and safe DNA-based plasmid immunization regimes. Moreover, it is reported that in *Sting*-deficient mice, host antitumor T cell responses was lost and rejection of immunogenic tumors was ablated. However, recently study revealed that STING deficient mice were resistant to 7,12-dimethylbenz(a) anthracene (DMBA) skin tumorigenesis (Ahn et al., 2014), which may suggest a role of facilitating tumorigenesis of STING in cancer. Further research of positive and negative regulation of the STING pathway and the role of STING plays in the cancer context should lead to new therapeutic strategies against cancer. In particular, STING agonists could be considered for a potential therapy target.

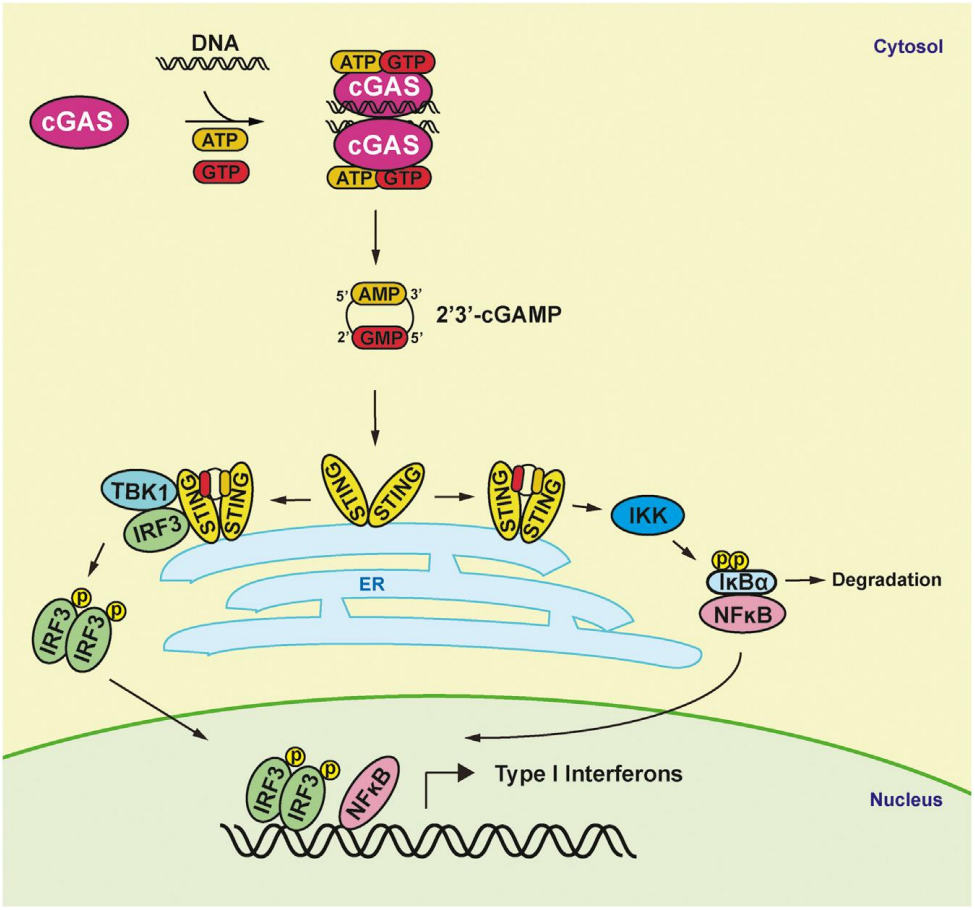

Figure 1.3 Overview of cGAS-STING pathways
Cytosolic DNA activates cGAS to form a cGAS-DNA complex with formation of 2'3'-cGAMP from ATP and GTP. 2'3'-cGAMP further binds and activates STING through structural activates. Activated STING phosphorylate IRF3 by recruitment of TBK1. IRF3 subsequently translocates into nucleus along with NF-κB to induce type I IFNs and other cytokines production. Image taken from Cai et al., 2014.

1.5 Clustered regularly interspaced short palindromic repeats (CRISPR)

A number of genome editing technologies have emerged in recent years, including zinc-finger nucleases (ZFNs) (Porteus and Baltimore, 2003), transcription activator-like effector nucleases (TALENs) (Zhang et al., 2011) and the RNA-guided CRISPR-Cas nuclease system (Deveau et al., 2010; Horvath et al., 2010). While ZFN and TALEN are used as strategies of tethering endonuclease catalytic domains to modular DNA-binding proteins

for inducing targeted DNA double-strand breaks (DSBs) at specific genomic loci. In CRISPR, a nuclease called Cas9 is guided by small RNAs through Wastson-Crick base pairing with target DNA (Garneau et al., 2010; Jinek et al., 2012) (Figure 1.4). CRISPR is a system that is significantly easier to design, highly efficient and specific and well suited for multiplexed gene editing for a variety of cell types and organisms.

Cas9 promotes genome editing by stimulating a DSB at a target genomic locus efficiently (Urnov et al., 2010; Hsu et al., 2012). Upon cleavage by Cas9, the target locus usually undergoes two major pathways for DDR: the error-prone NHEJ or the high-fidelity HDR pathway, both of which can be employed to achieve an expected editing outcome. In the absence of a repair template, DSBs are reconnected through the NHEJ process, which leaves scars in the form of insertion or deleting mutations. NHEJ can be useful to mediate gene knockouts, as deletion happening within a coding exon can lead to reading frame shift mutations and premature stop codons (Perez and Zhang, 2012). Multiple DSBs can be exploited to mediate larger deletions in the genome. HDR is an alternative major DNA repair pathway. HDR typically happens at lower and substantially more variable frequencies than NHEJ, but it can generate precise, defined modifications at a target locus in the presence of an exogenously introduced repair template. Unlike NHEJ, HDR is commonly active only in dividing cells, and its efficiency varies widely depending on the cell type and state, as well as the genomic locus and repair template

(Saleh-Gohari and Heeleday, 2004).

1.5.1 Overview of the CRISPR-Cas system

CRISPR-Cas is a microbial adaptive immune system that employs RNA-guided nucleases to cleave foreign genetic species (Deveau et al., 2010). There are three types (I-III) of CRISPR systems have been discovered and identified among a wide range of bacterial and archaeal hosts. Each system comprises a cluster of CRISPR-associated (Cas) genes, noncoding RNAs and a distinctive array of repetitive elements (direct repeats). These repetitive elements are interspaced by short variable sequences derived from exogenous DNA targets known as protospacers, and they constitute the CRISPR RNA (crRNA) array together (Makarova et al., 2011) Within the DNA target, each protospacer is associated with a protospacer adjacent motif (PAM), which can vary depending on the specific CRISPR system (Marraffini and Sontheimer, 2008; Brouns et al., 2008).

In this study, the type II CRISPR system was employed. Type II CRISPR system includes the nuclease Cas9, the crRNA array encoding the guide RNAs (gRNAs) and a required auxiliary transactivating crRNA (tracrRNA) that facilitates crRNA array processing into discrete units (Garneau et al., 2010). Each crRNA contains a 20-nt guide sequence and a partial direct repeat, where the former sequence leads Cas9 to 20-bp DNA target by Watson-Crick base

pairing (Figure1.4). In this system, the target DNA must precede a 5'-NGG PAM, where as other Cas9 orthologs may have different PAM requirements, such as those of S. thermophiles (5'-NNAGAA for CRISPR1 and 5'-NGGNG for CRISPR 3) and Neisseria meningiditis (5'-NNNNGATT) (Cong et al., 2013; Garneau et al., 2010).

The CRISPR-Cas RNA-guided nuclease function is reconstituted in mammalian cells by the heterologous expression of human codon-optimized Cas9 and the requisite RNA components (Cho et al., 2013). Moreover, the crRNA and tracrRNA can be formed to a fusion to create a chimeric, single-guide RNA (sgRNA) (Jinek et al., 2012). Cas9 can thus be redirected toward nearly any target of interest in vicinity of the PAM sequence by altering the 20-nt guide sequence within the sgRNA (Jinek et al., 2012). Due to its ease of implementation and multiplexing capacity, Cas9 has been used to generate engineered eukaryotic cells carrying specific mutations via both NHEJ and HDR (Hwang et al., 2013). Direct injection of sgRNA and mRNA encoding Cas9 into embryos has enabled the rapid generation of transgenic mice with multiple modified alleles (Wang et al., 2013; Shen et al., 2013).

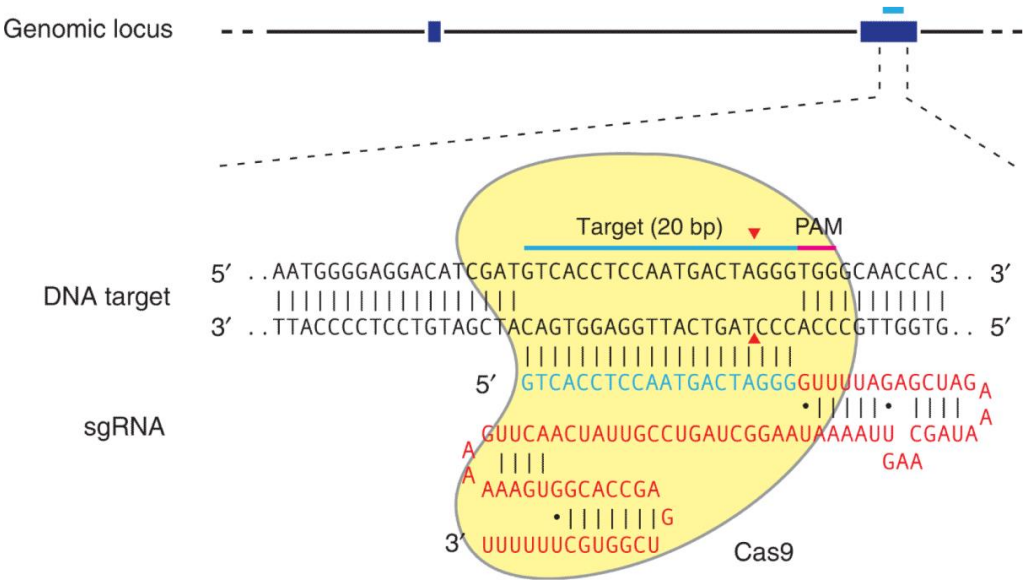

Figure 1.4 Overview of CRISPR-Cas9 system

Cas9 nucleases (yellow) are targeted to genomic DNA by an sgRNA consisting of 20 nucleotides length guide sequence (blue) and a scaffold (red). The guide sequence pairs with target DNA (blue bar), where upstream of a requisite 5'-NGG adjacent motif (pink) directly. Cas9 mediates a double strand break 3bp upstream of PAM (red triangle). Image taken from Ran et al., 2013.

1.6 Aims

Previous report indicated that STING is highly mutated in cancers suggesting a role for STING in cancer (Sauer et al., 2011). The aim of this project was to investigate the role of STING and type I IFN signaling pathway in cancer.

Chapter 2

Materials and Methods

Cell lines

A549 and HeLa cells were cultured in DMEM (Invitrogen, Singapore) supplemented with 10% FCS (Hyclone, USA), and 1% pen/strep (Invitrogen, Singapore). Wild type (WT) HCT116 cell lines were a generous gift from K. Miyagawa (Hiyama et al., 2006). Cells were cultured in McCoy (Invitrogen, Singapore) supplemented with 10% FCS (Hyclone, USA) and 1% pen/strep (Invitrogen, Singapore). FCS was heat inactivated for 30 mins at 50 ℃ before addition to cell culture media. All cells were grown at 37°C in a humidified 5% CO_2 incubator (Thermo Scientific, Singapore).

Western Blot

Whole cell extracts were prepared from purified cells from different cell lines and electrophoresis in 10% SDS-PAGE gels, and blotted onto nitrocellulose membranes (BioRad). For the preparation of whole-cell extracts, cells were lysed in Radio-Immunoprecipitation Assay (RIPA) buffer consisting of 150mM NaCl, 1mM EDTA, 50mM Tris-HCL (pH7.4), 1% NP-40 and 1% sodium deoxycholate (Sigma). In addition, protease inhibitor cocktail set III and phosphatase inhibitor cocktail set V (Merck Millipore, Germany) were added to the lysis buffer according to the manufacturer's instructions. Antibodies and GAPDH (Sigma) specific antibodies and horseradish peroxidase-coupled second stage reagents were used to develop the blots (Thermo). Blots were exposed on X-ray film (Fuji), and densitometry analysis

was performed using ImageJ 1.46. Analysis of blots was done by MetaMorph, which is a professional software for imaging analysis.

Reagents

Aphidicolin, Ara-C and dimethyl sulfoxide (DMSO) were purchased from Sigma (Singapore); cyclic dinucleotides (CDNs) were purchased from Invivogen;

Transfection and Transduction

For transfection and transduction, retroviral supernatants were generated as described (Diefenbach et al., 2003). Briefly, to prepare retroviruses, 293T cells were transfected with: 1) retroviral MSCV-GFP vectors 2) packaging plasmids encoding structural proteins of retroviruses, env, gag and pol contained in pFB vector along with 3) Transfectin (Biorad, USA). Transfection was performed in a 6-well plate and left to incubate at 37℃ for 48 h. Cell culture media was changed 4 h after transfection. The retroviral supernatant generated after 48 h was passed through a 0.45 μm filter (Satrorius Stedium, Germany) into target cells for transduction. Cells were spun at 2,000 rpm for 90 mins at 34℃. Upon 48 h after transduction, the top 10% of GFP-positively expressing cells were sorted using MoFlo sorter (Beckman Coulter, USA) and subsequently expanded for experiments, microscopy, real-time PCR or ELISA.

Generation of STING knockout cell lines

STING knockouts were generated in A549, HeLa and HCT116 Cell lines using the CRISPR/Cas9 system. Target gRNA expression constructs were selected from human gene database containing target RNA sequences listed in Table S1 cloned into gRNA cloning vector (Addgene). 8 x 10^6 cells were transfected with 6 µg Target gRNA and 6 µg pSpCas9(BB)-2A-GFP (Addgene) in a 10 cm tissue culture-treated plate. 36 h post-transfection, cells were single-cell sorted into 96-well tissue culture-treated plates and allowed to grow up about 2 weeks. The following primers were used: *STING*-5': tttcttggctttatatatcttgtggaaaggacgaaacaccgcgggccgaccgcatttggg; *STING*-3': gactagccttattttaacttgctatttctagctctaaaaccccaaatgcggtcggcccgc

Quantitative Real Time PCR

Total RNA was isolated using the Nucleospin RNA II kit according to manufacturer's instructions (Macharey Nagel, Germany) and reverse transcribed using M-MLV Reverse Transcriptase (Promega, USA) with random primers (Promega, USA). The total reaction was 25 µl made up of reverse transcribed RNA, 0.2 µM forward primer, 0.2 µM reverse primer and 12.5 µl of iTaq SYBR Green Supermix with ROX (Bio-Rad, Singapore). Triplicates were performed for the PCR reaction using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Singapore). The thermocycling parameters were 50 ℃ (2 min), 95 ℃ (3 mins) followed by 40 cycles of 95 ℃ (15 sec), 60 ℃ (30 sec) and 72 ℃ (45 sec). Finally, samples were normalized to the signal generated using housekeeping gene HPRT.

Samples prepared without total RNA was served as negative controls. The following primers were used: *HPRT*-5': gctataaattctttgctgacctgctg; *HPRT*-3': aattacttttatgtcccctgttgactgg; *STING*-5': ggctttagccgggaggatag; *STING*-3': gcaggttcctggtaggcaa; *IFNα4*-5': agaggccgaagttcaaggtta; *IFNα4*-3':tgtgggtctgaggcagatca; *IFNβ*-5': aaactcatgagcagtctgca; *IFNβ*-3': aggagatcttcagtttcggagg. Two-tailed unpaired t-test (GraphPad) was applied for statistical analysis. $P<0.05$ denotes significance.

Microscopy

For PicoGreen staining, cells were treated with 6 μM PicoGreen (Life technology, Singapore) for 1-2 h at 37℃. After treatment, cells were fixed with 4% formaldehyde for 10 mins. And then, cells were washed with PBS three times before addition of 2 mg/ml of RNase A (Sigma, Singapore) for 1 h at 37℃ to ensure that only DNA-specific signals are observed. Slides were washed once in PBS before mounting with Dako fluorescent mounting medium (Dako, UK).

For DNA antibody staining, cells were fixed with 4% formaldehyde for 10 mins. DNA was denatured using 50% Formamide (VWR International, USA) in PBS for 10 min at room temperature followed by incubation for 20 min at 75℃. Cells were washed with PBS three times before addition of 2 mg/ml of RNaseA (Sigma, Singapore) for 1 h at 37℃ to ensure that only DNA-specific signals are observed. For double-stranded DNA (dsDNA) staining, the

controls included pretreatment with 100 U/ml DNase (Sigma, Singapore) for 1 h at 37 ℃. Cells were washed with PBS and blocked with 1% BSA in PBS for 1 h. For ssDNA stainings, some cells were incubated with 200 U/ml S1 nuclease (Thermo Fisher Scientific) for 1 h at 37 ℃. After washing with PBS, cells were stained with ssDNA (clone F7-26, Merck) or dsDNA (MAB1293, Merck)-specific antibodies, followed by anti-mouse IgG coupled to Cy3 (AP124C, Merck), or anti-mouse IgM coupled to Cy3 (AP128C, Merck) antibodies. Stained cells were stained with DNA fluorochrome DAPI (0.5 μg/ml in PBS, #71-03-01, KPL Inc., USA) for 10 min. Slides were washed once in PBS before mounting with Dako fluorescent mounting medium (Dako, UK).

Chapter 3

STING Regulates Levels of Cytosolic DNA in Cancer Cells

3.1 Generation of STING knock out (KO) cells

STING was previously shown to be crucial in type I IFN mediated signaling pathway (Lemos et al., 2014). We therefore investigated the role of STING in different cancer cells. Firstly, we employed CRISPR to knock out STING in cancer cells to generate STING-deficient cells. Cas9-GFP plasmid and CRISPR construct plasmid were co-transfected into A549, HeLa and HCT116 cells. After 24 hours, GFP positive cells were single cell sorted into 96-well plates. Protein lysate was extracted from cell cultures derived from sorted cells. No STING was detected in some of the sorted cells (Figure 3.1a). Furthermore, STING signals were not observed in the same $STING^{CRISPR}$ cells when analyzed by confocal microscopy (Figure 3.1c). In summary, our data show that we successfully generated STING-deficient A549, HeLa and HCT116 cells.

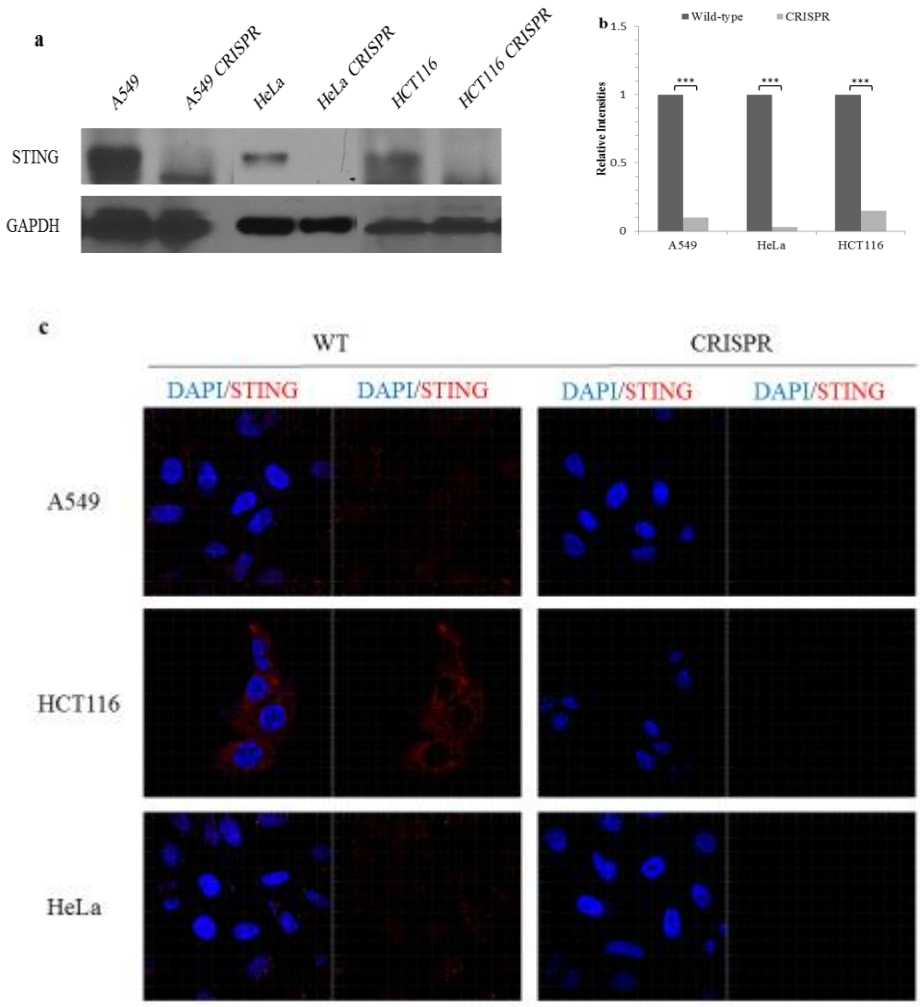

Figure 3.1 Generation of STING deficient cells by CRISPR.

(a) Western blot analysis of A549, HeLa and HCT116 cells transfected with control or STING-specific CRISPR constructs. Immunoblots were probed with antibodies specific for STING and GAPDH; (b) Quantification of relative STING expression. Intensities of STING bands were determined by Metamorph and normalized to GAPDH levels and relative STING levels in control transfected cells. ***$p<0.005$; (c) Control and STING CRISPR transfected A549, HCT116 and HeLa cells were stained with STING-specific antibody (red) and DAPI (blue).

3.2 Decreased levels of cytosolic DNA in STING-deficient A549 cells

To test whether the presence of STING affects the accumulation of cytosolic DNA, we stained cells with antibodies specific for ssDNA, dsDNA and DNA:RNA hybrids. Surprisingly, we found that the levels of cytosolic DNA significantly decreased in the different STING-deficient cells (Figure 3.2a and 3.2b).

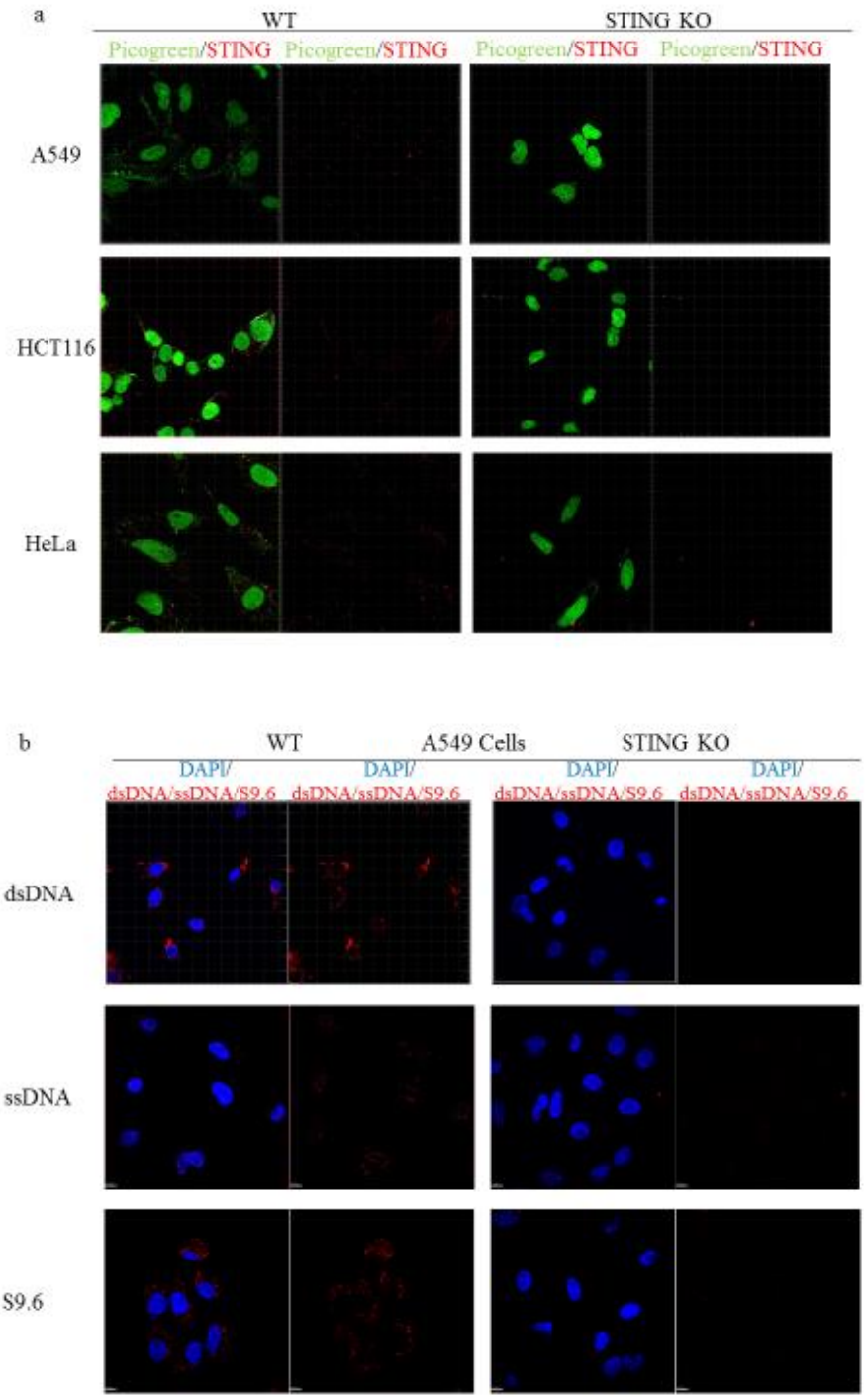

Figure 3.2 STING deficiency decreases the levels of cytosolic DNA in cancer cells.

(a) WT and STING deficient cells were stained with 6 μM dsDNA-specific vital dye PicoGreen for 2 hours; (b) A549 cells and A549 STING KO cells were stained with dsDNA, ssDNA or DNA:RNA hybrid (S9.6)-specific antibodies. Cells in the indicated panels were treated with 2 mg/ml RNaseA for 1 hour before staining.

3.3 The levels of cytosolic DNA do not increase in STING-deficient cells in response to DNA damage

We previously found that levels of ssDNA and dsDNA increased in the cytosol after treatment with the genotoxic agent Ara-C (Lam et al., 2014). However, we observed that STING-deficient cancer cells failed to accumulate cytosolic DNA in response to Ara-C (Figure 3.3). The result suggests a key role of STING in the accumulation of cytosolic DNA in response to DNA damaging agents.

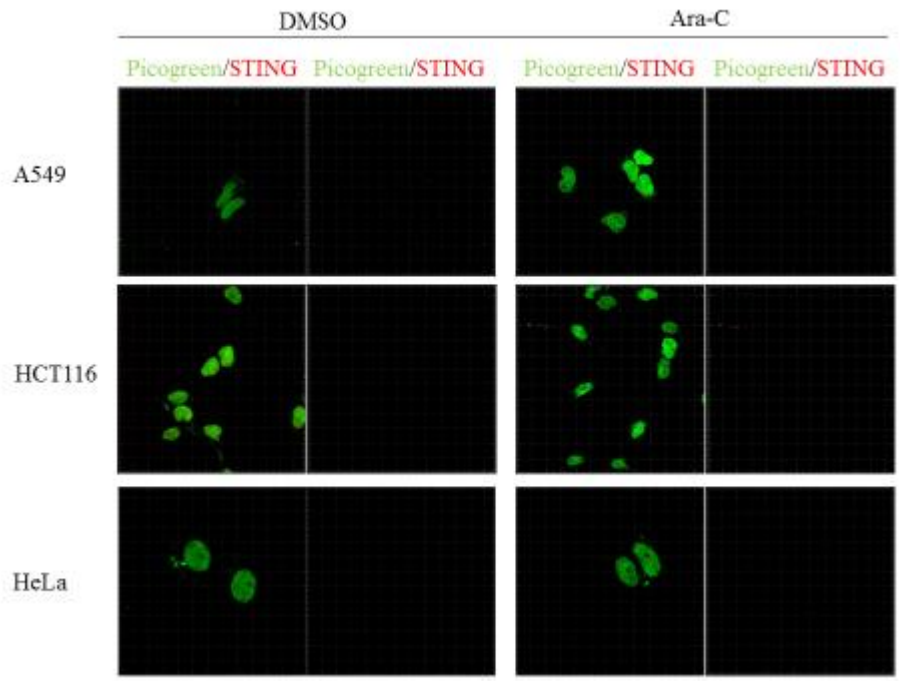

Figure 3.3 The levels of cytosolic DNA do not increase in STING-deficient cells in response to Ara-C.

A549, HCT116 and HeLa cells were stained with 6 μM dsDNA-specific vital dye Picogreen for 2 hours (green) after 10 μM Ara-C treatment or DMSO treatment for 16 hours. Cells in the indicated panels were treated with 2 mg/ml RNaseA for 1 hour before staining.

Chapter 4

STING is Crucial to Production of Type I IFNs in Cancer Cells

4.1 STING deficiency decreases the level of Type I IFNs in cancer cells

Type I IFNs play a critical role in a range of immune responses, including the promotion of antitumor activity (Cho and Kelsall, 2014). STING is reported to be crucial for type I IFN expression in normal cells, hence we analyzed if STING contributes to the expression of type I IFN in cancer cells. As shown in Figure 4.1, there was a significant decrease in type I IFNs expression level in STING deficient cells. IFNα4 and IFNβ were downregulated by 8.3 and 19.2-fold in A549 STING KO cells, and 5.6 and 7.7-fold in HCT116 STING KO cells respectively. The results suggest that STING is an important regulator of type I IFN expression in cancer cells.

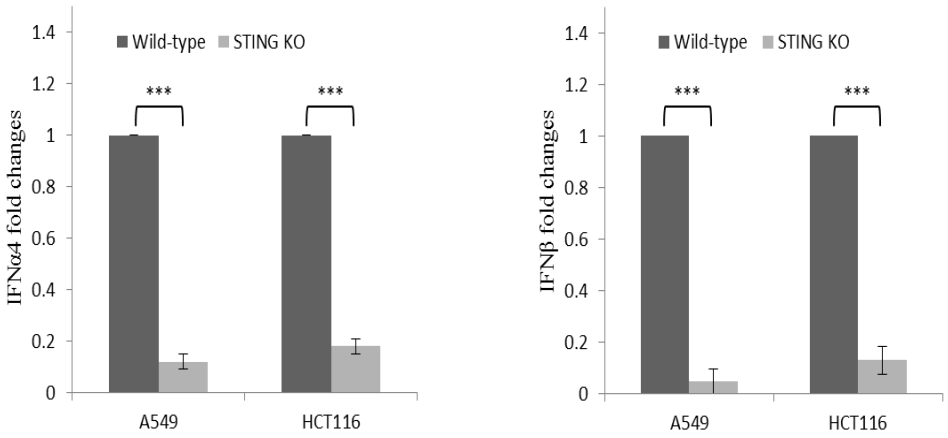

Figure 4.1 STING contributes to type I IFN expression in cancer cells.
Relative IFNα4 and IFNβ transcriptional levels in WT (black column) and STING-deficient (grey column) A549 and HCT116 cells were measured by quantitative real-time PCR. Data are mean +/- standard deviation of biological replicates. ***$p<0.005$, n=3

4.2 The levels of type I IFNs do not increase in STING-deficient cells in response to DNA damage

We previously found that DNA damaging agents such as aphidicolin induce significant secretion of type I IFNs. We therefore investigated whether the increase of type I IFN in response to DNA damage depends on STING. We treated WT and STING-deficient cells with aphidicolin for 6 hours, and analyzed type I IFNs expression level. After 10 μM Aphidicolin treatment, IFNα4 was upregulated by 6.3 and 7.4-fold, while IFNβ was upregulated by 6.8 and 5.3-fold respectively compared to DMSO treatment (Ctrl). However, there was no significant change of type I IFNs expression in STING-deficient cells (Figure 4.2). The levels of type I IFNs do not increase in STING-deficient cells, while increased significantly in WT cells in response to Aphidicolin. This result suggests that type I IFNs secretion is induced by DNA damaging agents in a STING dependent manner.

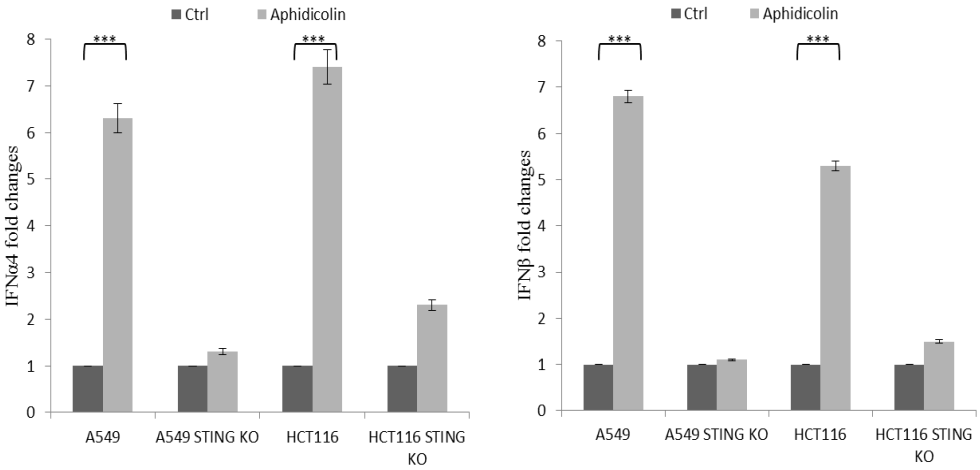

Figure 4.2 The levels of type I IFNs do not increase in STING-deficient cells in response to aphidicolin.

Relative IFNα4 and IFNβ transcriptional levels in WT (black column) and STING-deficient (grey column) A549 and HCT116 cells were measured by quantitative real-time PCR. Data are mean +/- standard deviation of biological replicates. ***p<0.005, n=3

4.3 Exogenous dsDNA does not induce type I IFNs expression in STING deficient cells

DNA damaging agents induce the presence of cytosolic DNA (Lam et al., 2014). Cytosolic DNA triggers the innate immune response leading to type I IFN expression (Cho & Kelsall, 2014). Consistent with previous reports, we found that STING is required for the expression of type I IFNs in A549 and HCT116 cells as WT, but not STING-deficient cells in upregulation of type I IFNs upon transfection of DNA. Hence, these results also confirm that we successfully generated STING-deficient cells.

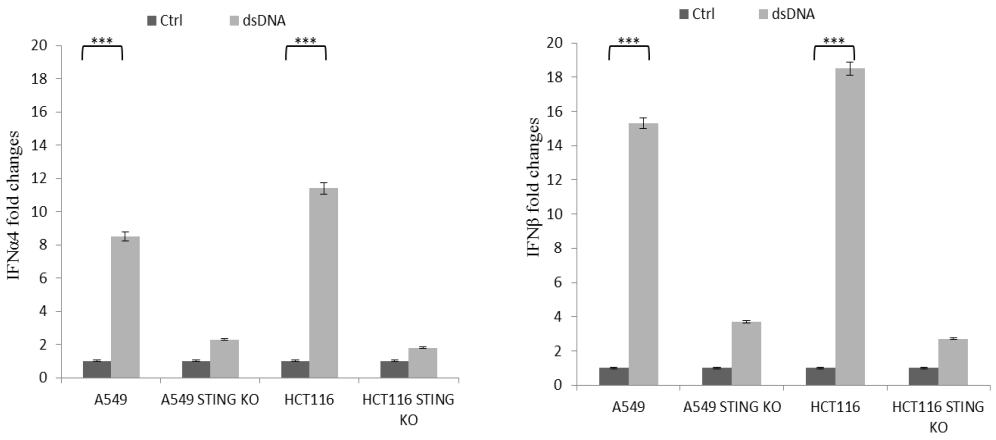

Figure 4.3 The levels of type I IFNs do not increase in STING-deficient cells in response to transfection of DNA.
Relative IFNα4 and IFNβ transcriptional levels in WT (black column) and STING-deficient (grey column) A549 and HCT116 cells were measured by quantitative real-time PCR. Data are mean +/- standard deviation of biological replicates. ***p<0.005, n=3

Chapter 5

Cyclic Dinucleotides (CDNs) Can Trigger Type I IFNs Expression in HEK293T Cells

5.1 The levels of type I IFNs do not increase in respond to dsDNA transfection in 293T cells.

A recent report indicates that type I IFN pathway is blocked by a splice variant of STING, which is termed as MITA-related protein (MRP), in 293T and HeLa cells, while 293T and HeLa cells can express endogenous STING. (Chen et al., 2014). Therefore, we analyzed the levels of type I IFNs upon dsDNA transfection in these two cell lines. The levels of type I IFNs increased significantly in respond to dsDNA transfection in HeLa cells, but not in 293T cells (Figure 5.1). After dsDNA transfection for 6 hours, IFNα4 and IFNβ transcript levels were upregulated by 13.6 and 15.8-fold respectively in HeLa cells. However, there was no significant change of type I IFNs expression in 293T cells. These results suggest that dsDNA cannot induce type I IFN production in 293T cells

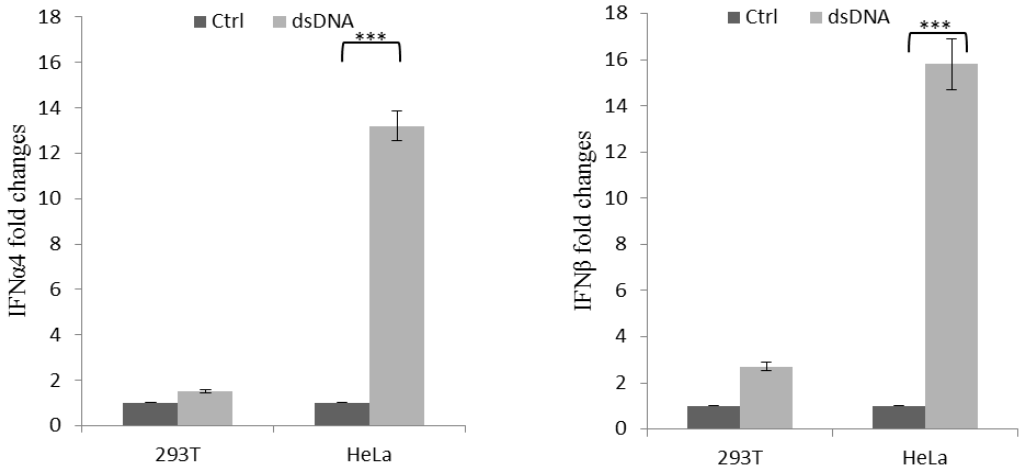

Figure 5.1 dsDNA transfection induces type I IFN production in HeLa cells but not 293T cells.

Relative IFNα4 and IFNβ transcriptional levels in WT (black column) and STING-deficient (grey column) 293T and HeLa cells were measured by quantitative real-time PCR. Data are mean +/- standard deviation of biological replicates. ***$p<0.005$, n=3

5.2 CDNs induce the expression of type I IFNs in 293T cells

CDNs are ubiquitous second messenger molecules used in bacterial signal transduction and act as defense triggers in mammalian cells (Wu et al., 2013). Upon bacterial infection, CDNs are released in the cells to activate STING and type I IFN production. Cyclic diguanylic acid (c-di-GMP) is the most prevalent intracellular signaling intermediate in bacteria. Other important CDNs include cyclic diadenylic acid (c-di-AMP) and cyclic adenylicguanlic acid (cGAMP). In this study, we used 2' 3'-cGAMP to investigate if MRP blocks type I IFN response. Surprisingly, 293T cells secret type I IFN upon stimulation with CDNs. After CDNs treatment for 6 hours, IFNα4 and IFNβ was upregulated by 16.7 and 20.3-fold respectively. However, there was no significant change of type I IFNs expression after transfection of dsDNA. These results suggest that CDNs can trigger type I IFN pathway in 293T cells, which further demonstrated that type I IFN response is not blocked in 293T cells.

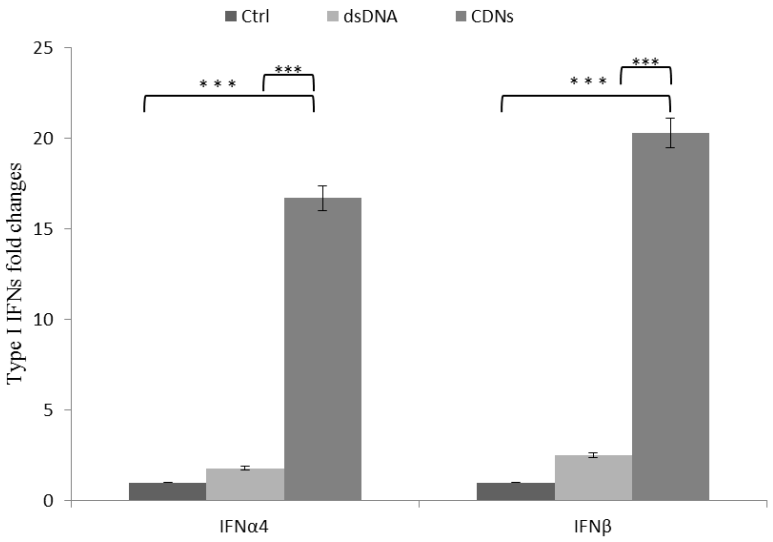

Figure 5.2 CDNs but not dsDNA induce type I IFNs expression in 293T cells. Relative IFNα4 and IFNβ transcriptional levels in 293T cells were measured by quantitative real-time PCR in response to dsDNA (light grey) and CDNs (deep grey). Data are mean +/- standard deviation of biological replicates. ***p<0.005, n=3

5.3 CDNs induce the presence of cytosolic DNA in cancer cells in a STING-dependent manner

To further investigate the reason for type I IFN activation by CDNs, we analyzed changes that cells respond to CDNs. By staining with antibody specific against dsDNA, we found that CDNs increase the levels of cytosolic dsDNA in the cytosol. However, in STING-deficient cells, the levels of cytosolic DNA did not increase in response to CDNs stimulation (Figure 5.3). These results suggest that the presence of cytosolic DNA is induced in a STING-dependent manner by CDNs.

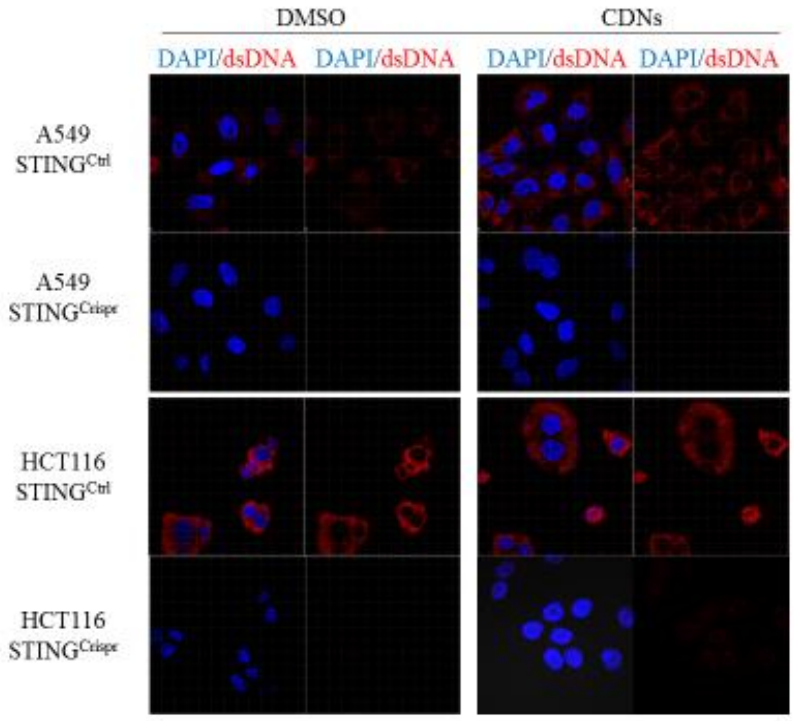

Figure 5.3 CDNs induce the presence of dsDNA in the cytosol of cancer cells.
WT and STING-deficient A549 and HCT116 cells were stained with dsDNA specific antibodies (red) and DAPI (blue) after treatment with 1 μg/mL CDNs for 16 hours. Cells were treated with 2mg/ml RNaseA for 1 hour before staining with dsDNA-specific antibodies.

Chapter 6

Discussions and Future Perspectives

6.1 Summary of key findings

In the present study, we investigated the role of STING in cancer cells. Our results show that STING may regulate the presence of cytosolic DNA. Furthermore, we provide evidence that STING is important for the constitutive low expression of type I IFNs cancer cells.

To investigate the link between STING and type I IFNs pathway, we used 293T cell line, of which type I IFNs are reported to be blocked by MITA-related protein (MRP). By stimulation of CDNs, we found that the levels of type I IFNs increased significantly comparing with dsDNA stimulation in 293T cells. Our findings suggest that type I IFNs pathway is not blocked in cancer. In summary, our study reveals a novel role for STING in cancer innate immunity.

6.2 Regulation of expression of type I IFN by STING in cancer cells

6.2.1 Regulation of type I IFNs production by STING

Previously, we found that genotoxic stress induces type I IFNs activates immune responses (Bist et al., 2013). Given that genotoxic stress is highly related to infections and cancer, our findings suggested a new mechanism for the regulation of immune system such as type I IFNs pathway in cancer cells. It is reported that STING is an important regulator for type I IFNs expression under DNA damage, but the details are remaining unclear.

Here we investigated the role of STING, which is a core cytosolic DNA sensor that can recognize cytosolic DNA and RNA introduced by microbes or viruses (Palm and Medzhitov, 2009). Previous studies provide evidence that DDR could activate STING expression, therefore induces IRF3 phosphorylation and type I IFNs production (Burdette and Vance, 2013), but the details of these processes need to be investigated.

The DDR was found to be constitutively activated in many cancer cell and precancerous tumor samples (Weber & Ryan, 2014). Consistent with these findings, we found that Yac-1 tumor cells contain detectable cytosolic DNA and exhibit constitutive activation of STING/TBK1/IRF3 (Lam et al., 2014). Cancer cells express relatively low level of type I IFNs with the activation of STING/TBK1/IRF3, which might be due to STING mutations reported to be prevalent in many cancer cells.

As previously reported, *STING* was found to be mutated in many cancer cells (data not shown). For that reason, we characterized the role of STING in cancers. An interesting finding in our lab was that cells subjected to DNA damaging agents can accumulate DNA in the cytosol, which suggests that cytosolic DNA is a possible trigger for DNA damage-dependent activation of TBK1/IRF3 and ultimately the production of type I IFNs (Lam et al., 2014). It is well established that DNA sensor pathways can activate STING/TBK1/IRF3 pathway (Stetson and Medzhitov, 2006). Strikingly, our data suggest that the production of type I IFNs critically depends on STING. Hence, DNA sensors

that activate STING, such as cGAS are possibly required for constitutive type I IFN expression as well as type I IFN response to DNA in cancer cells. How DNA damage and the DNA damage response affect the DNA accumulation in cell cytosol and the following biological response remains to be clarified and elucidated in more details. It is possible that DNA repair of genomic DNA damage leads to the presence of cytosolic DNA.

It remains unclear how STING is activated in response to DNA damage. It is possible that the DDR contributes more directly to STING activation by phosphorylating important residues in these molecules. *Atm*-deficient mice prime the type I IFN response via the STING pathway, and loss of ATM leads to the presence of cytosolic ssDNA (Härtlova et al., 2015). It is possible that CHK1 or other DDR effector kinases downstream of ATR play a crucial role in STING activation. Our finding shows that STING may regulate the presence of cytosolic DNA, and more details need to be established of the link between cytosolic DNA, STING and type I IFNs in cancer cells.

Taken together, DNA damage in cancer cells can lead to the presence of cytosolic ssDNA, dsDNA and DNA:RNA hybrids. These cytosolic nucleic acids are recognized by DNA sensor pathways that activate STING-TBK1-IRF3 pathway and initiate an innate immunity response.

6.2.2 Nature of cytosolic DNA in tumor cells

Cytosolic DNA has been shown to accumulate in cells under viral and

bacterial infection or uptake of apoptotic host cells (Ishii and Akira, 2006). We found the presence of different kinds of cytosolic DNA presence in uninfected human tumor cells. Firstly, an interesting question is where cytosolic DNA originates from and the how cytosolic DNA accumulates in tumor cells.

There are several studies that report the release of mitochondria DNA (mtDNA) in the cytoplasm upon cellular stress, which is a possible source for cytosolic DNA accumulation. During apoptosis, the secretion of oxidized mtDNA could activate NLRP3 inflammasome, leading to the production of IL-1β. IL-5 or IFN-γ primed eosinophils could release mitochondrial DNA on reactive oxygen species dependent manner, but independent of eosinophil death and the release of DNA happened rapidly in a catapult-like manner (Yousefi et al., 2008). Neutrophils were also found to release mitochondrial DNA in a similar way. However, it is unclear if mtDNA includes dsDNA, ssDNA and DNA: RNA hybrids present in the cytosol.

One possible explanation is that cytosolic DNA is derived from genome. Our lab previously provided evidence that cytosolic DNA in mice cells is derived from genomic DNA (Shen et al., 2015). Our data indicate that cytosolic dsDNA is generated during the DDR-dependent DNA repair of retroelements (Hedges and Deininger, 2007). Retroelements, inverted repeats and long tandem repeats can result in replication fork stalling and homologous recombination that can result in deletion of genomic DNA.

It is interesting to investigate more details about the role of cytosolic DNA in cancer cells. In HEK293T cells, it is reported that the endogenous expression of MITA-related proteins (MRP) will affect STING normal function, and further block the type I IFN response upon DNA infection. However, we found that 293T cells are able to secrete type I IFNs upon the stimulation of cyclic denucleotides (CDNs), which are ubiquitous second messenger molecules that can directly bind to and activate STING. This result suggests that cytosolic DNA in 293T cells fails to activate spontaneous type I IFN mediated signaling pathway. One possible explanation is cytosolic DNA in infected cells is different from cancer cells that cannot be detected by cGAS to trigger the STING pathway. It would be interesting to elucidate the difference between cytosolic DNA in cancer cells and infected cells, which may provide a potential cancer therapeutic pathway.

Recognition of DNA in the cytoplasm happens in a sequence independent and length dependent manner (Hornung and Latz, 2010). However, DNA sequence may possible affect the level of induction of type I IFNs. The homo-copolymer poly (dA:dT) was found to be the strongest activator of IRF3 transcription while other homo-copolymers such as poly (dG:dC) or poly (dI:dC) were much less potential IFN inducers (Ishii et al., 2006). Thus, the study of length and sequence of cytosolic DNA might provide insights into the difference in cancer cell cytosolic DNA and the efficiency of STING activation.

DNA isolated from viruses, bacteria or mammals appears to be recognized

equally well when transfected into responsive cells, as long as the DNA fragments have sufficient length (Ablasser et al., 2009). The characterized cytosolic DNA sensors have been reported to bind DNA directly; however, the binding specificities and affinities of only few putative sensors have been determined. Crystal structure of the IFI16 HINb domain was found to be sequence independence of DNA recognition when forming a complex with DNA, and the binding occurs at the sugar-phosphate backbone of DNA specifically (Jin et al., 2012). Another example is cGAS, which has a similar sequence-independent interaction in the structure (Civril et al., 2013). However, further study need to be conducted to establish more details about dsDNA fragments, such as the minimum length and why a minimum length of dsDNA fragments are needed to induce a type I IFN response in cells.

Beside viral infection, nucleic acids are described to be key activators for the innate immunity systems in several situations. Intracellular DNA is a crucial PAMP during intracellular bacterial infection, such as Listeria monocytogenes (Rathinam et al., 2010), Mycobacerium tuberculosis (Manzanillo et al., 2012), protozoan Plasmodium falciparum (Sharma et al., 2011), and Franscisella tularensis (Atianand et al., 2011). Moreover, in autoimmune diseases, for example SLE, DNA can also act as endogenous signal during conditions of sterile inflammation (Ablasser et al., 2013). DNA from dead cells which is not effectively diminished by DNases, such as DNase I in the extracellular space, DNase II in lysosomes and three prime repair exonuclease 1 (TREX1) in the

cytoplasm, can trigger DNA sensing pathways. Similar with other DNA, DNA from dying cells can act as a 'danger' signal in immune response to the vaccine adjuvant alum (Marichal et al., 2011). Similarly, our data suggests that presence of cytosolic DNA in tumor cells is important for the constitutive expression of type I IFN and may act as an endogenous DAMP.

6.2.3 Role of STING in cancer

STING is a 379 amino acid transmembrane containing protein that resides as a dimer in the endoplasmic reticulum (ER) of epithelial, endothelial cells as well as a variety of hematopoietic cells such as macrophages and dendritic cells (DCs) (Konno & Barber, 2014). STING has been found to be essential for triggering the production of various cytokines including type I IFNs in response to the detection of pathogen related dsDNA in the cytosol of the cells, or CDNs such as cyclic di-AMP produced from intracellular bacteria. Moreover, it has recently been reported that STING could possibly be responsible for causing many auto-inflammatory diseases, such as systemic lupus erythematosus (SLE) by becoming activated by intracellular DNA. However, mammalian STING is known to weakly bind to dsDNA species but can be strongly activated by CDNs that are generated from bacteria such as Listeria monocytogenes (Witte et al., 2012). The other type of CDN is non-canonical CDNs, such as cyclic di-GMP-AMP, which are generated under

the presence of ATP and GTP (Burdette et al., 2011). These CDNs can bind to STING dimers and activate STING-TBK1 pathway, which is a autophagy-related trafficking from ER to lysosomal/endosomal perinuclear regions that contain transcription factors IRF3 and NF-κB (Ishikawa et al., 2009). STING functions as an adaptor and key sensor in the DNA induced type I IFN response. STING is shown to play a pivotal role in controlling a variety of inflammation-driven biological activities, and possibly control MyD88-dependent carcinogen-induced skin cancer. Recently study revealed that STING deficient mice developed colonic tumors at a higher frequency compared to normal mice (Ahn et al., 2015). However, the role of STING in tumorigenesis has not been elucidated, but STING was found to contribute to apoptosis and inhibition of B cell lymphoma cells proliferation, and STING has been shown to be responsible for triggering vascular and pulmonary syndrome. Given the crucial role of STING in type I IFN mediated signaling pathway in a variety of DNA virus infection, STING shows a potential to act as a tumor suppressor by preventing infection of cells by oncoviruses (Heiber and Barber, 2012).

By generating STING deficient cancer cells, we investigated the role of STING in regulating the expression of type I IFNs. As mentioned in the introduction, type I IFN was reported to play in important role in immunosurveillance of tumors (Woo et al., 2015). Our data suggest that deficiency of STING blocks type I IFN expression in response to transfected

DNA. Strikingly, STING also plays an important role in the regulation of type I IFNs expression in cancer cells. Further experiments are needed to investigate and establish the role of STING in cancer. A possible experiment could be to cross *Sting*-deficient mice to Eu-*Myc* mice, a mouse model for B-cell lymphoma and analyze the cytokines expression levels.

We found the lower cytosolic DNA presence in STING deficient cancer cells. Given the importance of cytosolic DNA presence in cancer and DNA damage response, the reason for its decrease need to be further studied. A possible explanation is STING can stabilize the existence of different kind of cytosolic DNA, so STING deficiency leads to cytosolic DNA degradation. To understand this, a chromatin immunoprecipitation (ChIP) for STING can be applied. And the result will help us to understand more details of cytosolic DNA, such as the sequence and structure.

Several articles reported that an alternatively spliced isoform of STING, which is termed as MITA-related protein (MRP), had been identified in HeLa cells and HEK293T cells (Chen et al., 2014). The first 253 amino acids portion of MRP is identical to MITA, containing the dimerization domain but lacking the TBK1-binding domain. MRP acts dominantly as a dominant negative mutant of MITA and blocks STING-mediated IFN induction by TBK1-IRF3 by disrupting the STING-TBK1 interaction (Chen et al., 2014). This finding provides a new insight for STING regulation on type I IFN signaling in cancer. To investigate more details about the role of MRP in the STING pathway, we

analyzed the level of type I IFNs in 293T cells upon the stimulation of CDNs and dsDNA. However, we found that 293T cells secrete type I IFNs upon the stimulation of CDNs but not dsDNA. Given that CDNs can directly bind to STING, the results suggested that the STING pathway is not blocked by MRP in cancer cells. We also found that type I IFNs are able to be responsive to dsDNA in HeLa cells but not in 293T cells. This is possibly because the different ratio of MRP to STING in these cells, which means STING in 293T cells is completely blocked by MRP, while there are some functional STING in HeLa cells. It is also possible that there are different STING mutations in 293T and HeLa cells. Further experiments are needed to characterize details of STING in these two cells lines, which can help us understand the interaction between MRP and STING. Because dsDNA fail to trigger the type I IFNs secretion, a possible reason for the dysfunction of type I IFN pathway is upstream of STING mediated pathway, such as cGAS deficiency on DNA recognition. Because CDNs can increase the level of cytosolic DNA presence, another possibility is the cytosolic DNA induced by CDNs is different from dsDNA that cannot trigger type I IFNs production. The result also suggested a new pathway for STING mediated pathway that CDNs could not only directly bind to STING, but also affect cGAS by induce cytosolic DNA accumulation. Further studies will be needed to be conducted to characterize cGAS and its recognition of dsDNA in different cancer cells.

6.3 Future work

In light of the phenotype that we observed in STING-deficient cancer cells, more experiments are needed to understand the relationship between STING and cytosolic DNA accumulation in cancer cells. Our data suggest that, STING is an important adaptor molecule that links the pathway from cytosolic DNA sensors such as cGAS to downstream cytokines such as type I IFNs. It would be necessary to cross *Sting*-deficient mice with Eu-*Myc* mice, and further evaluate cytokines expression levels.

An important question is the mechanism of MRP in cancer cells. It would be worthwhile to identify potential interacting partners of MRP. By comparing MRP with STING, this can provide insights into the mechanism of MRP and STING regulation over type I IFNs mediated signaling pathway as well as other immune innate activities such as NF-κB expression. In addition, because genome instability is one of the cancer hallmarks, it will be interesting to investigate the role of STING in the DDR (Hanahan and Weinberg, 2011).

It would also be worthwhile to study if loss of MRP could lead to the normal function of type I IFN pathway in human cancer cell lines. With the highly efficient CRISPR system, it is possible to utilize the system to generate MRP-deficient cells to do further research.

6.4 Conclusion

Here we investigated the potential role of STING in cancer cells. Our findings

suggest that STING is important for presence and sensing of cytosolic DNA in cancer cells. We provide evidence that the STING pathway is defective in some cancer cells lines. Hence the restoration of STING activity in cancer cells might be a novel promising approach to the treatment of cancer.

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