Merkel Cell Polyomavirus Small T Antigen Perturbs the Cellular DNA Damage Response

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Merkel Cell Polyomavirus Small T Antigen Perturbs the Cellular DNA Damage Response

A dissertation presented

by

Rosa Yoon

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

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Merkel Cell Polyomavirus Small T Antigen Perturbs the Cellular DNA Damage Response

Abstract

Merkel cell polyomavirus (MCPyV) small T antigen (ST) is expressed in the majority of Merkel cell carcinomas (MCC), a highly lethal and aggressive cancer of the skin. Since the discovery of MCPyV in 2008, the role of ST in the context of the virus and MCC has been under intense investigation. Much of our knowledge of polyomavirus ST comes from research on other polyomaviruses, including mouse polyomavirus (MPyV) and simian virus 40 (SV40). Both MPyV and SV40 ST contribute to transformation in part by binding to and inhibiting the cellular phosphatase PP2A. Likewise, MCPyV ST interacts with PP2A, although mutants that are reported to abolish this interaction still transform cells, suggesting that MCPyV ST has PP2A-independent functions. Understanding the unique cellular perturbations induced by MCPyV ST will thus be important for understanding the tumorigenesis of MCC.

In this dissertation, we sought to understand the manipulation of cellular functions by MCPyV ST. We began by characterizing the MCPyV ST protein itself, starting with structural and functional comparisons with other well characterized polyomaviruses and identifying the interaction of MCPyV ST with cellular proteins. We observed that MCPyV ST uniquely interacts with the TIP60 cellular complex, which contains an ATPase and an acetyltransferase and is involved in histone modifications and DNA damage repair. Through predictions of the structure,
we identified a surface-exposed region of ST, loop 4, and observed that regions in this loop were important for regulating the binding of ST to the TIP60 complex.

Functionally, we investigated the role of MCPyV ST in the DNA damage response because of its interaction with TIP60 and because DNA damaging agents are used to treat MCCs. In addition, overcoming checkpoint regulation in the p53 pathway is an open question in MCPyV infection. We determined that ST increased sensitivity to DNA damage by γ-irradiation and etoposide and that expression of ST caused persistence of double strand DNA breaks (DSB) after damage, suggesting that DSB repair was delayed in ST expressing cells. Specifically, we observed that ST expression inhibits repair of breaks by nonhomologous end joining (NHEJ) but does not inhibit repair by homologous recombination (HR). These effects on the DNA damage response are explained in part by a less robust phosphorylation of DNA-PKcs at serine residue 2056, which is important for regulating end processing and repair by NHEJ. Taken together, these results indicate that MCPyV ST disrupts the cellular DNA damage response, which has implications on the viral life cycle and the initiation and treatment of MCC.
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Chapter I. Background
The Polyomaviridae family

Polyomaviruses are small double-stranded DNA viruses, which have been intensively studied due to their effects on a number of cellular processes, including DNA replication and cell cycle regulation. Historically, these viruses were of particular interest because the initially discovered viruses of the family, mouse polyomavirus (MPyV) and simian virus 40 (SV40), were observed to cause transformed phenotypes in cell culture and cancer in animals under some conditions. The detection of SV40 in monkey cell lines used to produce poliovirus vaccines led to intense investigation of the potential oncogenic effect of SV40 on humans (Sweet and Hilleman, 1960). However, there is no convincing evidence implicating SV40 in human disease (reviewed in Poulin and DeCaprio, 2006).

Polyomaviruses were first implicated in human disease with the discovery of BK polyomavirus (BKPyV) from urine and JC polyomavirus (JCPyV) from brain samples in the 1970s (Gardner et al., 1971; Padgett et al., 1971). BKPyV and JCPyV are pathogenic in individuals with weakened immune systems and are related to diseases of the kidney and to Progressive Multifocal Leukoencephalopathy (PML), respectively (Arthur et al., 1986; Koralnik et al., 1999; van der Meijden et al., 2010). Additional human polyomaviruses have been discovered more recently, including KI polyomavirus, WU polyomavirus, human polyomavirus 6, human polyomavirus 7, trichodysplasia spinulosa-associated polyomavirus (TSV), and Malawi polyomavirus (MWPyV) (Allander et al., 2007; Gaynor et al., 2007; Schowalter et al., 2010; Siebrasse et al., 2012). Merkel cell polyomavirus (MCPyV) was the first polyomavirus characterized as being associated with human cancer (Feng et al., 2008).
The polyomaviruses are highly prevalent in human populations. The human polyomaviruses BKPyV, JCPyV, MWPyV, TSV and MCPyV have high overall seroprevalence with seroprevalence increasing with age [Berrios et al., 2015; van der Meijden et al., 2011; Viscidi et al., 2011]. MCPyV titers were also observed to be positively correlated with age, which has implications for human disease [Viscidi et al., 2011].

**Merkel Cell Polyomavirus and Merkel Cell Carcinomas**

MCC is a rare but aggressive form of skin cancer with approximately 1500 new cases per year [Lemos and Nghiem, 2007]. Like melanoma, MCC is associated with UV exposure [Miller and Rabkin, 1999]. MCC occurs more frequently in patients with immunosuppression upon organ transplantation, with acquired immunodeficiency syndrome (AIDS), and in the elderly [Engels et al., 2002]. Due to these factors, MCC had been suspected to be of infectious origin long before the identification of MCPyV.

MCPyV was initially identified by digital transcriptome subtraction (DTS), using cDNA libraries derived from MCC tumors that were compared against known sequences [Feng et al., 2008]. The MCPyV genome was subsequently isolated and sequenced from MCC tumors. MCPyV sequences were observed to be clonally integrated in some of these tumors, which points to a causal role for the virus [Feng et al., 2008]. In addition, MCPyV DNA and expression of viral proteins have been reported in many MCC tumors tested [Feng et al., 2008; Garneski et al., 2009; Rodig et al., 2012; Shuda et al., 2008]. MCPyV is thought to contribute to the formation of MCC; however, MCPyV infection is not sufficient for the formation of MCC (reviewed in [Chang and Moore, 2012]).
T antigens and MCC

Polyomavirus T antigens are expressed from the early region of the viral genome, which is transcribed before viral DNA replication. In MCPyV, this region includes the splice variants ST, LT, and some truncated forms of LT antigen, and Alternate frame of the Large T Open reading frame (ALTO) (Figure 1.1). ALTO results from a frameshift of the second LT exon [Carter et al., 2013]. Other variants of the T antigen exist in other polyomaviruses. MPyV, for example, expresses middle T antigen (MT). The polyomavirus T antigens are multifunctional proteins that are important for viral replication, gene expression, and alteration of host cell cycle.

MCC tumors express viral T antigens. Importantly, MCC tumors express a mutated form of large T antigen (LT) that is prematurely truncated in exon 2 and does not include the helicase portion of LT and a full length small T antigen (ST) [Shuda et al., 2008; Shuda et al., 2011]. The mutations in LT were observed to inhibit the ability of LT to facilitate viral genome replication [Shuda et al., 2008]. The expression of T antigens in MCC tumors was initially observed to be necessary for the growth and survival of MCPyV positive MCC tumor cell lines [Houben et al., 2010]. LT knockdown is thought to cause cell death [Shuda et al., 2011]. It was originally reported that ST is important for growth of established MCC cell lines [Shuda et al., 2011]. However, later reports have shown that ST is not necessary for MCC growth [Angermeyer et al., 2013, 2014]. Thus, the role of ST in the maintenance of MCC remains unclear.
Figure 1.1: MCPyV Early Region. The MCPyV early region encodes for splice variants of the MCPyV T antigen, including ST, LT, an alternative spliced 57kT, and ALTO. MCC tumors express full length ST and various prematurely truncated LT variants (Adapted from (Cheng et al., 2013) and (Carter et al., 2013)).
Small T Antigen

ST is a smaller splice variant of the T antigen that has high sequence conservation among polyomaviruses (Figure 1.1). ST is composed of a conserved DNA J domain and unique domain that is not present in LT. Much of our understanding of polyomaviruses ST derives from studies of SV40 and MPyV and forms the basis for potential hypotheses regarding the function of MCPyV ST.

The small T antigens are involved in cellular transformation. SV40 ST has been reported to play a role in cellular transformation in combination with LT antigen and other cellular factors (Bikel et al., 1987; Hahn et al., 2002; Sleigh et al., 1978; Yu et al., 2001). Similarly, MPyV MT and ST have been shown to cooperate to fully transform cells (Asselin et al., 1983; Asselin et al., 1984; Noda et al., 1986).

One of the best characterized conserved interactions of ST is with protein phosphatase 2A (PP2A). PP2A is a holoenzyme composed of the scaffolding subunit A, the regulatory subunit B and the catalytic subunit C (Janssens and Goris, 2001). By binding to PP2A, ST is able to alter the catalytic activity of PP2A (Yang et al., 1991). The interaction between ST and PP2A has been shown to contribute to cell cycle progression in both mouse polyomavirus (MPyV) and SV40. Notably, SV40 ST is thought to bind only to PP2A Aα whereas MPyV binds to both PP2A Aα and Aβ (Andrabi et al., 2011; Zhou et al., 2003). This results in functional differences between SV40 and MPyV ST (Andrabi et al., 2011). MCPyV ST also can bind PP2A but the ability of MCPyV ST to transform may be independent of PP2A binding or DnaJ binding activities (Shuda et al., 2011). The effect of ST on cells, therefore, may vary despite the similarities between these proteins.
The cellular DNA damage response

Correctly repairing DNA breaks is essential for maintaining the integrity of the genome. The DNA damage response (DDR) involves a signaling cascade that connects the detection of a break by sensors and the subsequent repair by effector molecules. The DDR is essential for preventing genomic instability, a key driver of carcinogenesis.

In mammalian cells, double strand breaks (DSB) can be formed in many ways. For example, DSB are formed in meiosis to increase genetic diversity and in the rearrangements of immunoglobulin and T cell receptor genes in the process termed V(D)J recombination [Gellert et al., 1999; Whitby, 2005]. In addition, DSB can form as a consequence of a replication fork colliding with an unrepaired single strand break (SSB) during cellular replication [Strumberg et al., 2000]. DSB are also formed from reactive oxygen species (ROS) produced as a result of normal cellular metabolism, exposure to ionizing radiation, and can be induced by some chemotherapeutic agents, such as the topoisomerase inhibitor etoposide [Ross and Bradley, 1981; Wozniak and Ross, 1983].

Key steps in DSB signaling

The repair of DSB is more difficult to orchestrate than other types of DNA damage repair. Erroneous repair of DSBs can lead to the loss of genetic material, amplification, or chromosomal translocations [Khanna and Jackson, 2001], which can lead to cancer, senescence or apoptosis. The initiation of DSB repair and checkpoint regulation is thought to be mediated by three kinases, ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related protein (ATR) and DNA-dependent protein kinase (DNA-PK), which form a group called the phosphatidylinositol
3-kinase-related kinase (PIKK) family. Signaling by ATM, ATR and DNA-PK leads to the recruitment of mediators of DNA damage repair and activation of the DNA damage checkpoint (reviewed in Durocher and Jackson, 2001).

In the DDR, the first step is the recognition of the damage. Though the initial steps happen very quickly, the MRN complex (MRE11-RAD50-NBS1) is thought to be one of the first molecules to recognize the break (Uziel et al., 2003). The MRN complex then recruits DNA damage proteins to the site of a break (Williams et al., 2007).

Another key early step in the DDR is the activation of ataxia telangiectasia mutated (ATM), which is a serine/threonine kinase. ATM activation is related to its interaction with the MRN complex and changes in chromatin structure around the site of the break (Bakkenist and Kastan, 2003; Uziel et al., 2003). Specifically, ATM is activated through multiple protein modification steps. Inactive ATM dimers are activated through dissociation and trans-autophosphorylated at serine 1981 (Bakkenist and Kastan, 2003). ATM activity is modulated by a number of proteins. For example, PP2A negatively regulates ATM autophosphorylation at S1981; thus, the regulation of PP2A may be important for regulating subsequent ATM activity (Goodarzi et al., 2004). Other post translational modifications of ATM are important for ATM activity after DNA damage. These include ribosylation by PARP1 and acetylation at K3016 by Tip60 (Aguilar-Quesada et al., 2007; Sun et al., 2007; Xu et al., 2011). ATM activity is required for the downstream phosphorylation of multiple targets including p53, MDM2, CHK1, CHK2, BRCA1, and NBS1 (Khanna and Jackson, 2001).

The ATM-mediated phosphorylation of histone H2AX at S139, referred to as γH2AX, occurs rapidly after damage (Burma et al., 2001; Rogakou et al., 1998). γH2AX rapidly
accumulates around the site of a DSB, forming detectable foci, which are predictive of the total number of DSB within a nucleus (Paull et al., 2000; Rogakou et al., 1999). Though the phosphorylation of H2AX was thought to be regulated only by ATM, it has been reported that DNA-PK, ATM, and ATR can each phosphorylate H2AX at S139 (Stiff et al., 2004). Prevention of formation of γH2AX led to a decrease in recruitment of the DNA damage proteins RAD51 and BRCA1 (Paull et al., 2000), indicating that γH2AX is important for the recruitment of downstream mediators of DNA damage. Interestingly, DDR signaling factors including MRN, 53BP1, and BRCA1 are recruited at low levels to sites of DNA damage in H2AX null cells but do not form foci, implying that the role of γH2AX is to concentrate mediators of repair on the sites of the breaks (Bouquet et al., 2006).

**Chromatin remodeling at DSB**

Other effectors that are recruited to the site of DSB include chromatin remodelers, which enhance the access of DDR effector proteins to DSB and facilitate repair. The RNF8 and RNF168 ubiquitin ligases cause chromatin remodeling that contributes to the DDR. RNF8, a ubiquitin ligase, assembles at DSBs through its interaction with MDC1 and ubiquitinates the histones around the DSB (Mailand et al., 2007). RNF168 is thought to amplify the ubiquitination of histones at breaks. Inhibition of RNF8 and RNF168 affects the recruitment downstream mediators of the DDR including BRCA1 and 53BP1 (Doil et al., 2009; Huen et al., 2007; Mailand et al., 2007). Interestingly, recent work has shown that 53BP1 can read the H2AK15ub mark, which is mediated by RNF168 after damage, and that this mark is necessary for recruitment of 53BP1 to DSB (Fradet-Turcotte et al., 2013). Thus, RNF8 and RNF168 are
important chromatin remodelers that may also play a direct role in recruitment of downstream proteins.

In addition, the multi-protein Tip60 complex is involved in the opening of structured chromatin at the site of double-strand breaks (DSB) in DNA (Xu and Price, 2011). The human Tip60 complex includes the proteins ACTL6A (BAF53A), BRD8, DMAP1, EP400 (p400), EPC1, ING3, KAT5 (TIP60), MEAF6, MRGBP, RUVBL1, RUVBL2, TRRAP, and YEATS4 (GAS41). Chromatin modifications most likely occur fairly early in the DDR, as Tip60 depletion leads to loss of MRN and γH2AX foci (Chailleux et al., 2010). Because it is involved in the remodeling of chromatin around the break, the Tip60 complex is important for repair by both HR and NHEJ (Murr et al., 2006; Robert et al., 2006). Tip60 is also important for post-translational modifications on H2AX. Tip60-mediated acetylation of H2AX is important at early points in the DNA damage response (Ikura et al., 2007). In addition, acetylation of H4 by Tip60 may be necessary for downregulation of the effects of γH2AX after repair (Jha et al., 2008; Kusch et al., 2004).

**Repair of DSB**

In mammalian cells, the two major pathways for DSB repair are homologous recombination (HR) and non-homologous end-joining (NHEJ). These processes are partially complementary in repair of DSBs (Takata et al., 1998). The choice between HR and NHEJ is largely cell cycle dependent and also depends on the chemical complexity of the breaks. NHEJ can occur during any stage of the cell cycle, but HR normally occurs in S and G2 phase, when a sister chromatid, and therefore a complementary sequence, is present (Rothkamm et al., 2003).
Overall, NHEJ is the primary method of repair for DSB and only about 15% of all IR induced DSB are repaired by HR \cite{Beucher2009}. ATM is thought to be the key kinase in the regulation of HR and DNA-PK is thought to be important for repair of breaks by NHEJ \cite{Bensimon2011}.

NHEJ is more error prone than HR. Repair by NHEJ can cause mutations, deletions, and translocations resulting from end processing. To initiate NHEJ, the Ku70/80 heterodimer and DNA-PKcs bind to the DSB, forming the DNA-PK holoenzyme \cite{West1998,Yaneva1997}. DNA-PKcs alone can bind to DNA ends and have some activity without the Ku70/80 proteins \cite{Hammarsten1998}. However, the Ku proteins greatly increase the activity and affinity of DNA-PKcs to the DSB \cite{West1998}. The crystal structure of the Ku heterodimer bound to DNA supports the idea that Ku70/80 are important for holding DNA ends at a synapse, recruiting other NHEJ proteins, and allowing error to take place \cite{Walker2001}.

Autophosphorylation of the catalytic subunit DNA-PKcs is required, not only for the activation of the DNA-PK holoenzyme, but also for the subsequent steps in NHEJ \cite{Chan2002,Ding2003}. NHEJ is carried out by a number of proteins, including the nuclease Artemis, XLF, XRCC4, and DNA ligase IV, which mediate processing and repair of the break \cite{Lieber2008,Ma2002,Riballo2009,Roy2012}.

In contrast to NHEJ, HR is restricted to S and G2 phase of the cell cycle, where the homologous sequence of DNA is utilized for an increased fidelity of repair \cite{Rothkamm2003}. HR begins with end resection by the Mre11-Rad50-Nbs1 (MRN) complex and CtIP \cite{Bressan1999,Limbo2007,Takeda2007,Williams2007}. End
rescession leaves single stranded DNA overhangs, which are rapidly coated by replication protein A (RPA). BRCA2 then mediates the loading of RAD51 onto the RPA coated single strand ends, displacing RPA and allowing RAD51 mediated strand invasion into the sister chromatid. Gene conversion, the process by which the homologous DNA is copied into the broken strand, can occur from the homologous sequence on the sister chromatid in S and G2 phase or at lower frequencies from other homologous sequences within the genome. The association of BRCA1 and BRCA2 with RAD51 is important for proficient recombination and repair of breaks.

Viruses and DNA Damage Response

Viruses often perturb normal cellular processes in order to facilitate viral replication and propagation. Viral proteins can alter cellular pathways through interaction with various cellular proteins. Many DNA viruses have been reported to interfere with the cellular DNA damage response, resulting in altered DNA damage sensitivity.

In some cases, activation of the DNA damage response is necessary to promote productive viral infection. The small DNA viruses HPV, MPyV, and BKPyV have been reported to activate the ATM pathway to promote viral replication. HSV-1 infection activates the ATM signaling pathway. Cellular proteins that are involved in HR are observed in early HSV-1 replication sites and may contribute to the replication of the virus. ATM has also been reported as involved in the replication of another herpesvirus, the
human cytomegalovirus (HCMV) [E et al., 2011; Luo et al., 2007]. Most relevantly, it has been reported that MCPyV infection causes LT to co-localize with ATM and ATR; both kinases are important for MCPyV replication (Tsang et al, 2014).

In contrast, the normal DNA damage response can be deleterious for the replication of some DNA viruses. Thus, viral proteins can also block the activation of parts of the DNA damage response. For example, adenovirus causes the degradation and mislocalization of the MRN complex to prevent concatemerization of its genome [Boyer et al., 1999; Stracker et al., 2002; Weiden and Ginsberg, 1994]. Failure to block MRN results in multimeric adenoviral genomes, which can no longer be packaged in virions. KSHV vIRF1 blocks activation of ATM and p53 to negate the effects of the checkpoint response [Shin et al., 2006].

Viruses can alter proteins in the DNA damage response to regulate expression of viral proteins. For example, Tip60 was initially identified as human immunodeficiency virus-1 (HIV-1) TAT interacting protein. The Tip60 histone acetyltransferase is involved in transactivation of the HIV-1 promoter and is required for HIV-1 replication [Kamine et al., 1996]. In contrast, the degradation of Tip60 by adenovirus allows for transcription of the viral protein E1A, which is essential for transcription of adenoviral genes [Gupta et al., 2013].

Viruses have also been implicated in direct interference with DNA damage repair processes, such as non-homologous end joining (NHEJ) and homologous recombination (HR). Adenovirus causes the degradation of ligase IV, which is a protein involved in NHEJ [Baker et al., 2007; Mohammadi et al., 2004]. Adenovirus E4orf6 causes prolonged autophosphorylation of DNA-PKcs at T2609 after IR [Hart et al., 2005]. HSV-1 ICP0 expression causes the degradation of DNA-PKcs [Lees-Miller et al., 1996]. JCPyV has been reported to cause the
inhibition of DSB repair pathways by modulating the levels of Ku70/Ku80 [Darbinyan et al., 2004]. JCPyV T antigen also has been reported to inhibit HR, which reduces the fidelity of DNA repair [Reiss et al., 2006].

Overall, the viral literature is rich with examples of viruses that inhibit and enhance the cellular DNA damage response as part of their life cycle.

**Potential role of DNA damage in MCPyV infection**

In this dissertation, we address a potential role for MCPyV ST in modulating the cellular dsDNA damage response based on several clues related to the MCPyV life cycle. Firstly, polyomavirus LT binding to RB can override cell cycle checkpoints and trigger a cell cycle checkpoint response [Dahl et al., 2005]. In the case of SV40 LT, binding to p53 can inhibit the ability of p53 to induce p21 and the checkpoint response [Lilyestrom et al., 2006]. In contrast, MCPyV LT is unable to bind to p53 and there was no apparent mechanism for how MCPyV could overcome this checkpoint [Cheng et al., 2013]. Since most MCC contain WT ST and maintain an intact p53, we suspected that ST could, in some manner, perturb the p53 dependent DNA damage response. Secondly, we discovered the interaction of ST with the Tip60 complex, which is a complex involved in chromatin remodeling and has been reported to play a key role in dsDNA damage repair [Sun et al., 2010]. Thirdly, integration into cellular DNA is not advantageous for MCPyV and in Merkel cell carcinomas (MCC), it is a non-productive pathway for the virus, as it involves truncation of LT and loss of viral replication [Shuda et al., 2008]. The probability of viral integration could be skewed to benefit the virus if ST interferes with the DNA damage response. Finally, MCC is highly responsive to therapeutics that induce DNA
damage, including cisplatin, etoposide, and irradiation. If the gene products of the early region can alter the DNA damage response, this points to a potential mechanism for the sensitivity of MCC. Therefore, understanding the function of viral proteins in MCC can provide a mechanism of action behind current clinical treatments.
Chapter II. Characterization of MCPyV ST antigen
Abstract

Merkel cell polyomavirus (MCPyV) small T antigen (ST) is a multifunctional protein that plays roles in viral infection and in Merkel Cell Carcinoma (MCC). We explored the interaction of MCPyV ST with two cellular complexes: the protein phosphatase 2A complex (PP2A) and the histone acetyltransferase Tip60 complex. To gain a better understanding of the potential roles of ST in both infection and in the formation and maintenance of MCC, we analyzed a predicted structure of ST by comparing it to the known co-crystal structure of SV40 ST and PP2A. Using this methodology, we were able to identify a region of ST that we call loop 4. Loop 4 is a structurally distinct portion of ST that could be involved in binding of other cellular proteins. Residues on loop 4 were responsible for the modulation of binding of ST to the Tip60 complex. Despite the published ST mutants that do not bind PP2A, we could not find a mutant that is unable to interact with PP2A while still maintaining Tip60 binding in our hands. This result suggests that PP2A binding could be responsible for either the structural integrity of ST or specifically for the binding of ST with the Tip60 complex. We also observed that adding epitope tags to ST had a severe effect on binding to the Tip60 complex but not the PP2A complex. Using epitope tags on ST could have an impact on its normal function in the cell due to changes in the ability to bind cellular proteins. Finally, we explored the localization of ST in the cell to help build hypotheses about ST functions in cells. We observed ST both in nuclear and cytoplasmic locations. Interestingly, the presence of ST altered the subcellular localization of Tip60. These results together suggest that MCPyV ST functions are unique from other polyomavirus ST and that the loop 4 region in MCPyV ST is critical for novel interactions.
Introduction

Polyomaviruses T antigens are expressed from the early region of the viral genome. In Merkel Cell Polyomavirus (MCPyV), this region includes small T antigen (ST), large T antigen (LT), some alternatively spliced forms of LT antigen such as 57kT, and Alternate frame of the Large T Open reading frame (ALTO) (Figure 1.1). ST is a smaller splice variant of the T antigen made up of the N terminal J domain and the C terminal unique region, which is not present in LT. All mammalian polyomavirus STs have conserved cysteine residues. For simian virus 40 (SV40) and mouse polyomavirus (MPyV) ST, these cysteine residues are required for PP2A binding [Chen et al., 2007b; Cho et al., 2007; Pipas, 1992].

The literature regarding polyomavirus-mediated transformation is predominantly from work with SV40 and MPyV. Functionally, SV40 ST has been reported to play a role in cellular transformation [Bikel et al., 1987; Hahn et al., 2002; Sleigh et al., 1978; Yu et al., 2001] and combinations of MPyV ST have been shown to augment transformation of cells by MT [Asselin et al., 1983; Asselin et al., 1984; Noda et al., 1986].

One of the best characterized conserved interactions of ST is with protein phosphatase 2A (PP2A), which is a holoenzyme consisting of the scaffolding subunit A, the regulatory subunit B and the catalytic subunit C [Janssens and Goris, 2001]. SV40 ST and MPyV ST have been shown to interact with the PP2A A and C subunits [Pallas et al., 1990; Walter et al., 1990]. SV40 ST binds directly to PP2A A whereas binding to the C subunit is likely mediated by the interaction with the A subunit [Ruediger et al., 1992]. SV40 ST is thought to bind only to PP2A Aα whereas MPyV ST binds to both PP2A Aα and Aβ [Andrabi et al., 2011; Zhou et al., 2003].
MCPyV ST has been reported to bind both PP2A Aα and Aβ (Griffiths et al., 2013; Kwun et al., 2015). By binding to PP2A, ST is able to alter the catalytic activity of PP2A (Yang et al., 1991). The binding site for the B subunit on the PP2A A subunit overlaps with the binding site for ST, suggesting that ST alters PP2A activity by replacing the B subunit in the complex (Chen et al., 2007b; Cho et al., 2007; Ruediger et al., 1992). It was shown that like other STs, MCPyV ST can displace the B subunit in the PP2A complex (Kwun et al., 2015). MCPyV ST displaces the B56α subunit but not the B55α, B56γ, B56δ, and B56ε subunits of PP2A, thereby affecting the specificity of the enzymatic activity (Kwun et al., 2015). In addition, the crystal structure of SV40 complexed to the PP2A subunit suggests that ST could interact directly with the active site of the C subunit and may be able to directly inhibit the activity of the C subunit (Cho et al., 2007).

The interaction of SV40 ST with PP2A has been shown to be important for transformation of cells by SV40 (Arroyo and Hahn, 2005). In contrast, MCPyV ST has been reported to transform cells independently of binding to PP2A (Shuda et al., 2011). This implies that cellular perturbations induced by MCPyV ST are distinct from those by SV40 ST, and points to the need to further understand additional host factors perturbed by ST.

MCPyV ST is expressed in Merkel cell carcinoma (MCC). MCC tumors express various truncations of LT and full length ST (Feng et al., 2008; Rodig et al., 2012; Shuda et al., 2008). In fact, the expression of a truncated MCPyV LT and ST in MCC tumors is necessary for the proliferation of MCPyV positive MCC tumor cell lines (Houben et al., 2010). Therefore, ST plays an important role in MCC.
In this chapter, we characterize MCPyV ST and ST mutants. Specifically, we examine the interaction of ST with PP2A and additional cellular proteins to address the differences between MCPyV ST and previously described polyomavirus STs. Mapping the interaction domains of MCPyV ST and cellular complexes, including PP2A, can help shed light on the important functional consequences of these interactions and provide clues to their contributions to MCC.

Contributions
The MudPIT experiments were performed by Dr. Jingwei Cheng in collaboration with the laboratory of Dr. Michael Washburn (Stowers Institute for Medical Research). Some mutants, shown in Figure 2.1 and Table 2.1, were cloned by Dr. Cheng and tested by both Dr. Cheng and myself. Also, some mutants in Table 2.1 were created by Christian Berrios, but tested by me. All other assays discussed in this chapter were designed and performed by me.

Materials and Methods
MudPIT, Multidimensional Protein Identification Technology (MudPIT) [Florens and Washburn, 2006] was performed by Jingwei Cheng in collaboration with the laboratory of Dr. Michael Washburn (Stowers Institute for Medical Research). MKL-1 and WaGa cells were lysed in EBC buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 0.5% NP40, 0.5 mM EDTA pH 8). Lysates were cleared by centrifugation at maximum speeds. Lysates were then immunoprecipitated overnight.
with Antibody 5 (Ab5) and Antibody 3 (Ab3) and washed in high salt EBC buffer (50 mM Tris HCl pH8, 300 mM NaCl, 0.5% NP40, 0.5 mM EDTA pH8). Cells were then submitted for MudPIT analysis. In order to generate a list of potential ST interacting proteins, the MudPIT analysis of samples immunoprecipitated with Ab5, which can immunoprecipitate both LT and ST containing complexes, was compared to the analysis of samples immunoprecipitated with Ab3, which can only immunoprecipitate LT containing complexes.

**Structure analysis and alignments.** Secondary structure prediction was generated by one-to-one threading of MCPyV ST with PDB structure 2pf4, the co-crystal structure of full-length SV40 ST complexed with the PP2A Aα subunit [Cho et al., 2007]. The predicted protein structure was generated with Phyre2 (Protein Homology/Analogy Recognition Engine) [Kelley and Sternberg, 2009]. Alignments of MCPyV ST and SV40 ST were performed using the Praline alignment algorithm [Simossis and Heringa, 2005].

**Cell lines and cloning.** Constructs were cloned into the Gateway vectors (Invitrogen) MSCV-N-Flag-HA-IRES-PURO (NTAP) or MSCV-C-Flag-HA-IRES-PURO (CTAP). Untagged constructs were expressed in the CTAP vector with a TAA stop codon to exclude expression of the epitope tag.

U2OS and 293T cells were grown in DMEM supplemented with 10% FBS, Glutamax and Pen Strep. To generate stable cell lines, retrovirus was generated in 293T cells. After transduction of U2OS cells overnight, cells were selected by splitting into media with puromycin (1 µg/mL).
To generate single cell cloned cell lines, U2OS stable cell lines were plated by serial dilution into 96 well plates. Each well was verified to contain a single colony by light microscopy. 10-15 wells were selected and expanded. Cells were tested by immunoblot for MCPyV ST to verify expression of the constructs.

**Immunoprecipitation and immunoblots.** Cells were harvested and washed in 1x PBS and lysed in EBC buffer (50 mM Tris HCl pH8, 150 mM NaCl, 0.5% NP40, 0.5 mM EDTA pH8) containing phosphatase and protease inhibitors (Calbiochem). Lysates were cleared by centrifugation at maximum speeds. Immunoprecipitation was performed overnight with Dynabeads incubated with Ab5 or p400 or with HA conjugated agarose beads (Roche). Beads were washed in high salt EBC buffer (50 mM Tris HCl pH8, 300 mM NaCl, 0.5% NP40, 0.5 mM EDTA pH8).

For fractionation of cells, cells were lysed first in Cytoskeleton (CSK) buffer (10 mM Pipes pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, and 0.5% Triton) to extract cytoplasmic proteins and lyse the plasma membrane. Lysates were cleared by centrifugation at maximum speeds. The resulting pellet was washed in CSK and lysed in EBC (50 mM Tris HCl pH8, 150 mM NaCl, 0.5% NP40, 0.5 mM EDTA pH8) to extract nuclear proteins. Lysates were cleared by centrifugation at maximum speeds. This pellet was washed with EBC buffer and subsequently lysed with RIPA (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% SDS, 1% NP40) and sonicated.

For immunoblots, lysates or immunoprecipitate was combined with 1x Laemmli buffer and run on 4-20% TGX gradient gels (Bio-Rad). Samples were transferred onto nitrocellulose membranes. Immunoblotting was performed with antibodies against Tip60 (Santa Cruz), TRRAP
(Bethyl), p400 (Bethyl), DMAP1 (Santa Cruz), Baf53A (Bethyl), PP2A Aα (Bethyl), PP2A Aβ (Bethyl), lamin (Cell Signaling), vinculin (Sigma), α-tubulin (Genetex), and ST (described in Cheng et al., 2013; Rodig et al., 2012). Membranes were blocked in 3% BSA and incubated overnight at 4° C in 3% BSA with primary antibody.

Growth assay. U2OS cells were counted and 50,000 cells were plated in 6 well plates. At various times, cells were stained and fixed with crystal violet (Sigma). Crystal violet stain was re-dissolved in 500 µL of 10% acetic acid. Subsequently, the absorbance at 590 nm was measured in a Tecan plate reader. Values were normalized to the values at 24 hours after plating.

Immunofluorescence microscopy. Cells were grown on glass coverslips in p60 plates. Cells were fixed in 10% neutral-buffered formalin solution (Fisher). Cells were permeabilized with 0.5% triton and 3% BSA in PBS for 30 minutes. Cover slips were incubated in primary antibody diluted in 3% BSA in PBS for 1 hour at room temperature and washed in PBS. Then, cover slips were incubated with secondary antibody diluted in 3% BSA in PBS for 30 minutes and washed. To stain nuclei, coverslips were incubated with DAPI (Invitrogen) for 10 minutes.

Results

ST interacting proteins

Previously, it was reported that MCPyV ST binds to protein phosphatase-2A (PP2A) (Shuda et al., 2011), which is a serine-threonine phosphatase made up of a scaffold subunit A,
the regulatory subunit B, and the catalytic subunit C. However, because there are functional differences reported between MCPyV ST and other polyomavirus STs, our laboratory decided to further investigate the cellular proteins that bind to MCPyV ST.

To identify other potential interacting proteins, Dr. Jingwei Cheng from our laboratory used Multidimensional Protein Identification Technology (MudPIT) in collaboration with the laboratory of Dr. Washburn (Stowers Institute for Medical Research). This technique combines chromatography with mass spectrometry to identify binding partners of proteins and determine the abundance of proteins in complexes. Samples for MudPIT analysis were prepared from lysates of MKL-1 and WaGa cells, two MCC-derived cell lines that contain integrated MCPyV genome and express ST and truncated LT.

By comparing the proteins that immunoprecipitated with Ab5, which binds to the J domain of ST and LT, and with Ab3, which binds to a unique region in LT, we were able to distinguish a number of proteins that bind to ST in MCC. As expected, immunoprecipitation of ST with Ab5 also pulled down members of the PPβA complex, PPβA AĮ, PPβA Aȕ, and PPβA CĮ. MCPyV ST binds both PPβA AĮ and PPβA Aȕ, which is similar to εPyV ST. This finding was also recently reported by other groups (Griffiths et al., 2013; Kwun et al., 2015). In addition, the MudPIT analysis revealed the interaction of ST with proteins in the Tip60 complex. The Tip60 complex proteins ACTL6A (BAF53A), BRD8, DMAP1, EP400 (p400), EPC1, ING3, KAT5 (TIP60), MEAF6, MRGBP, RUVBL1, RUVBL2, TRRAP, and YEATS4 (GAS41) were observed in the ST MudPIT analysis. The Tip60 complex is involved in chromatin remodeling and DNA damage repair (reviewed in Sapountzi et al., 2006; Sun et al., 2010).
To test the interaction between ST and Tip60 components, U2OS cell lines that stably express ST and selected mutant ST constructs were generated. In these cells, ST, but not truncated LT, is expressed and we are therefore able to directly test if the proteins identified in the MudPIT interact with ST. Retrovirus was generated in 293T cells using vectors expressing untagged ST, C-terminal FLAG-HA tagged ST (CTAP-ST), or N-terminal FLAG-HA tagged ST (NTAP-ST) (Figure 2.1A). The retroviruses were used to transduce U2OS cells, and cells were selected in puromycin for 2 weeks after transduction.

The interaction between ST and p400 was verified with immunoprecipitation followed by immunoblot. Initially, stable cells that express vector ST, CTAP-ST or NTAP-ST were immunoprecipitated with Ab5, which recognizes ST (Figure 2.1B). Immunoblots were performed with Ab5 and with antibodies against TRRAP and BAF53A, which are two members of the Tip60 complex (Figure 2.1B). Immunoprecipitation of untagged ST, CTAP-ST, and NTAP-ST with Ab5 showed co-immunoprecipitation of TRRAP and BAF53A.

A similar experiment was also performed with epitope tagged ST from cells that express vector, untagged ST, CTAP-ST, or NTAP-ST, using agarose beads conjugated to an antibody that recognizes the HA epitope tag. TRRAP and DMAP1 were detected in the immunoprecipitation in CTAP-ST cells, but not in the negative control vector only or the untagged ST expressing cells (Figure 2.1C).

The interaction was also detected in the reciprocal immunoprecipitation experiment. Cells were immunoprecipitated with an antibody against p400, followed by an immunoblot with Ab5 and antibodies against p400, DMAP1, and TIP60. ST could be immunoprecipitated with p400 (Figure 2.1D). As expected, p400 co-immunoprecipitated DMAP1 and TIP60, which are
other proteins in the Tip60 complex. Therefore, untagged ST and CTAP-ST interact with members of the Tip60 complex, which is consistent with the data from the MudPIT. Dr. Cheng was also able to reproduce the interaction of ST with the Tip60 complex in parallel experiments.
Figure 2.1: ST interacts with components of the Tip60 complex. (A) Diagram of constructs used in immunoprecipitation assays. Blue indicates FLAG-HA epitope tag in relation to the protein. Untagged ST containing a stop codon was expressed from the CTAP vector. U2OS cells were transduced with retrovirus to generate stable cell lines expressing vector, ST, CTAP-ST and NTAP-ST and selected in puromycin for 2 weeks. Immunoprecipitation from U2OS stable cell lines (B) Ab5, (C) anti-HA antibody, or (D) anti-p400 antibody. In Ab5 blots, the solid arrow indicates the expected size for untagged ST and the open arrow indicates the expected size of the FLAG-HA tagged ST construct.
Figure 2.1: ST interacts with components of the Tip60 complex (Continued).
**Effect of epitope tags on ST**

While wild-type untagged ST could interact with the Tip60 complex, NTAP-ST did not interact with the Tip60 complex in other experiments that the laboratory had previously performed. Whole cell lysates of cells containing the NTAP-ST construct expressed a strong band that is the expected size of NTAP-ST and a faster migrating band that was approximately the size of untagged ST (Figure 2.1B and 2.1C). One model to explain this result is that presence of a bulky epitope tag on the N terminus disrupts the shape of the ST protein. Therefore, I hypothesized that only untagged ST and CTAP-ST interact with this complex.

We decided to further investigate if NTAP-ST could bind to the Tip60 complex. U2OS cells that stably express vector, untagged ST, CTAP-ST or NTAP-ST were immunoprecipitated with anti-HA antibody. While the HA antibody immunoprecipitated both NTAP-ST and CTAP-ST, detection of the Tip60 components TRRAP and DMAP1 were only observed in the immunoprecipitation from CTAP-ST expressing cell lines (Figure 2.1C). Thus, the presence of an N-terminal tag, but not a C-terminal tag, interfered with ST binding to Tip60 complex ST and the Tip60 complex.

Performing the immunoprecipitation with Ab5 pulled down components of the Tip60 complex in all three ST-expressing cell lines, as described above. However, in the NTAP-ST cells only the Ab5, but not HA, immunoprecipitation pulled down Tip60 complex components. As Ab5 can pull down both untagged and tagged ST, it became apparent that the NTAP-ST construct also expresses a small amount of untagged ST, resulting in the detection of the Tip60 complex when the Ab5 immunoprecipitation was performed from NTAP-ST expressing cells.
To remove the expression of this small amount of untagged ST, we cloned a construct containing NTAP-ST with the first methionine mutated to an alanine (NTAP-ST-M1A). This mutant no longer co-immunoprecipitated Tip60 complex components when immunoprecipitated with either Ab5 or antibody against the HA epitope (Summarized in Table 2.1). Interestingly, though the N-terminal epitope tags disrupted binding to Tip60 complex components, NTAP-ST and CTAP-ST could still bind to PP2A (Summarized in Table 2.1)

This data suggests that epitope tags can disrupt the binding of ST with some cellular proteins and could therefore alter the results of biological assays. Specifically, N-terminal epitope tags seem to reduce the ability of ST to bind to the Tip60 complex and thus are likely to have an impact on certain functions of ST. Although CTAP-ST could still bind to Tip60 and PP2A in our immunoprecipitation assays, it does not rule out the possibility that the C-terminal epitope disrupts binding to other ST interacting proteins and thereby differ in its function from untagged ST. Therefore, though some of the early characterization of ST functions was performed with CTAP-ST, we decided to work primarily with untagged ST for functional assays to minimize any unknown effects from the presence of epitope tags.
Table 2.1: Summary of ST mutants and binding. ST and mutants cloned as either “tagged” or “untagged” constructs. Untagged constructs have a “TAA” stop codon. +: binding confirmed; -: no binding detected; +/-: impaired binding; ND: binding not determined; *: published function of a mutant that could not be reproduced in our lab. Tagged ST constructs are named CTAP or NTAP. Constructs without this notation have a C-terminal stop codon and therefore are untagged.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Vector</th>
<th>Tip60 Binding</th>
<th>PP2A Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTAP-SV40-ST</td>
<td>CTAP</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CTAP-ST</td>
<td>CTAP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CTAP-ST-D44N</td>
<td>CTAP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CTAP-ST-R7A</td>
<td>CTAP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CTAP-ST-L142A</td>
<td>CTAP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CTAP-ST-R102A</td>
<td>CTAP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NTAP-ST-M1A</td>
<td>NTAP</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>ST</td>
<td>CTAP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D44N</td>
<td>CTAP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R7A</td>
<td>CTAP</td>
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<td>+</td>
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<td>+</td>
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<tr>
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<td>CTAP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>W156A/ F157A</td>
<td>CTAP</td>
<td>-</td>
<td>-</td>
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Table 2.1: Summary of ST mutants and binding (Continued).

<table>
<thead>
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<th>Construct</th>
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<th>Tip60 Binding</th>
<th>PP2A Binding</th>
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<td>+/-</td>
</tr>
<tr>
<td>84A</td>
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<td>+</td>
</tr>
<tr>
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<td>CTAP</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>E86A/E87A</td>
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<td>-</td>
<td>+/-</td>
</tr>
<tr>
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<td>CTAP</td>
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<td>+</td>
</tr>
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<td>-</td>
<td>+</td>
</tr>
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<td>+</td>
</tr>
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<td>CTAP</td>
<td>-</td>
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<tr>
<td>E86S/E87S</td>
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<td>+</td>
</tr>
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<td>+</td>
</tr>
<tr>
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<td>-</td>
<td>+</td>
</tr>
<tr>
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<td>+</td>
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<tr>
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<td>+</td>
</tr>
<tr>
<td>S132A</td>
<td>CTAP</td>
<td>+</td>
<td>+</td>
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<td>H184(stop)</td>
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<td>+</td>
</tr>
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<td>+</td>
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<td>CTAP</td>
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<td>+</td>
</tr>
<tr>
<td>L136A</td>
<td>CTAP</td>
<td>+</td>
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Expression and localization of ST

Understanding the subcellular localization of ST can shed light into the functional roles of ST in MCC. After optimizing conditions for Ab5 for immunofluorescence microscopy, stable cells expressing NTAP-ST, CTAP-ST or untagged ST were fixed and prepared for immunofluorescence microscopy with Ab5 or with HA.

Interestingly, although untagged ST, CTAP-ST and NTAP-ST have differences in binding to cellular proteins, all three constructs were visible in the nucleus and cytoplasm by IF (Figure 2.2). NTAP-ST and CTAP-ST cell lines appear to have more distinct ST signal in the cytoplasm than untagged ST expressing cells. However, these apparent differences in staining intensity between the epitope tagged ST constructs and the untagged construct is likely due to the lower amount of untagged ST expression compared to expression of NTAP-ST and CTAP-ST (Figure 2.1), resulting in a lower signal to noise ratio, rather than being indicative of a difference in localization of untagged and epitope tagged ST.
Figure 2.2: ST is expressed in nuclear and cytoplasmic fractions when assessed by immunofluorescence microscopy. U2OS cell lines stably expressing vector, NTAP-ST or untagged ST were grown on cover slides, fixed in 10% neutral-buffered formalin solution (Fisher) for 30 minutes, permeabilized with 0.5% triton and 3% BSA in PBS for 30 minutes, and incubated with primary antibody against HA or Ab5 in 3% BSA for 1 hour at room temperature and secondary antibody in 3% BSA for 1 hour at room temperature. To stain nuclei, coverslips were incubated with DAPI (Invitrogen) for 10 minutes. (A) NTAP-ST cells with primary antibody against HA. (B) NTAP-ST cells incubated with Ab5. (C) CTAP-ST cell lines with HA. (D) Untagged ST cell lines with Ab5. The cell lines shown in this figure were not single cell cloned after transduction and selection and represent a pooled population of cells.
**Generation of clonal ST cell lines**

U2OS cell lines that stably express ST and selected mutant ST constructs had previously been generated for immunoprecipitation assays. In initial tests in functional assays, we observed that the variation in level of expression of ST across the various cells caused variability in phenotypes in clonogenic assays and by immunofluorescence microscopy. Therefore, I decided to create single cell clones of vector and ST cell lines. Generating single cell clones also had the advantage of allowing us to choose cells with the highest expression of ST.

After single cell cloning, 10 vector clones and 11 ST clones were selected and analyzed by immunoblot. As expected, the expression levels of ST in cells were highly variable between cells within the population (Figure 2.3A).

The single cell cloned cell lines were then assayed by growth assays. 10,000 cells were plated in 6 well tissue culture plates. At various time points during the assay, crystal violet staining was performed and the plates were assayed for relative cell density measuring the amount of crystal violet by spectrophotometry. Expression of untagged WT ST in cell lines did not significantly change the growth rate of the cells compared to vector (Figure 2.3B).
Figure 2.3: Single cell cloning of ST cell lines and growth of single cell clones. Pooled U2OS stable cell lines were plated by serial dilution into 96 well plates. Each colony was expanded and cell lines were selected for further analysis. (A) Single cell clones were tested by immunoblot for ST expression. Vinculin was used as a loading control. Vector cell lines 2 and 3 and ST cell lines
Figure 2.3: Single cell cloning of ST cell lines and growth of single cell clones (Continued).

6, 8, and 11 were selected for further analysis. (B) Growth assay of single cell clone cell lines expressing either vector or ST. Vector clones 2 and 3 and ST clones 6 and 8 were tested in each biological replicate, and plated in technical duplicates for each sample. Error bars indicate standard error of the mean across three biological replicates. Differences between vector and MCPyV ST were not significant when assessed with two-sided Student’s t-test (p > .05).
Predicted Structure of MCPyV ST

Though little is known about MCPyV ST structure and binding sites, there are two published crystal structures of SV40 ST co-crystalized with PP2A A and C subunits \cite{Chen2007, Cho2007}. These papers suggested that SV40 ST binds to the PP2A complex, displacing the PP2A B subunit and thus disrupting the normal cellular function of PP2A. I decided to further investigate the potential structure of MCPyV to better understand binding sites with both the previously identified PP2A and newly identified Tip60 complexes.

To identify potential domains that may regulate the interaction of MCPyV ST with cellular factors, I compared MCPyV ST to the crystal structures of SV40 in order to determine unique structural differences that would then help guide mutagenesis of MCPyV ST. A predicted structure of MCPyV ST was generated using Protein Homology/analogY Recognition Engine V 2.0 (Phyre2), a web-based algorithm for protein structure predictions \cite{Kelley2009}, manually inputting the crystal structure of SV40 as a model (Figure 2.4A).

By digitally overlaying the predicted structure of MCPyV ST with the SV40 crystal structure, we observed that MCPyV ST and SV40 ST are predicted to be structurally similar. In the predicted MCPyV ST structure, many of the reported SV40 ST-PP2A interaction sites could be conserved. This method also allowed for the identification of a region that we call loop 4, which had predicted structural dissimilarities with SV40 ST and was potentially a site for interaction with non-conserved proteins, such as members of the Tip60 complex (Figure 2.4A). This loop is on the opposite side of the PP2A A subunit binding face of ST. Interestingly, Kwun et al. used a similar modeling approach and described the LT-stabilization domain (LSD) of ST,
which is in the same region as loop 4 [Kwun et al., 2013]. Loop 4/LSD is predicted to be on the opposite molecular face from the residues that are thought to mediate the binding of ST to PP2A.
Figure 2.4: Predicted structure of ST and identification of Loop 4. (A) Phyre2 prediction of MCPyV ST structure in complex with PP2A A-alpha. This figure is displayed to highlight the relationship between the predicted MCPyV ST and PP2A A subunit. The PP2A A subunit is shown in grey. ST is shown in blue, with the “loop 4” region highlighted in pink. Loop 4 is on the opposite face of binding to PP2A A. (B) Praline alignment of SV40 and MCPyV STs. Stars indicate potential PP2A interacting residues based on Cho et al., 2007. The pink line indicates where loop4 is on our predicted crystal structure.
Characterization of ST mutants

PP2A binding mutants

Initially, we tested the published PP2A binding mutants R7A and L142A for binding to PP2A complex components. These mutants were reported to no longer have detectible interaction with PP2A Aα when assessed by immunoblot after co-immunoprecipitation [Shuda et al., 2011]. Stable cell lines expressing ST, R7A ST or L142A ST were immunoprecipitated with Ab5. PP2A Aα and Aβ were detected in the immunoprecipitate in all three ST constructs, but not in the vector control (Summarized in Table 2.1).

To identify other potential sites on MCPyV ST that may mediate the interaction with PP2A, protein alignments were used to identify conserved binding sites from SV40 ST. Based on the 3D structure analysis above, it seemed possible that the interacting residues between SV40 ST and MCPyV ST would be conserved in practice (Figure 2.4A). In addition to the previously reported R7 and L142, residues W156 and F157 were conserved between SV40 and MCPyV ST (Figure 2.4B). Therefore, the point mutants W156A, F157A, and the double mutant W156A/F157A ST were generated to test for binding to PP2A. These constructs were used to make stable cell lines with the CTAP retroviral vector and selecting cells with puromycin for two weeks. All three mutants expressed in these cell lines. Compared to wild-type MCPyV ST, the point substitution mutant W156A and the double point mutant W156A/F157A ST had impaired binding to PP2A Aα and PP2A Aβ. However, these mutants also had impaired binding to the Tip60 complex member TRRAP (Summarized in Table 2.1). The F157A single mutant retained binding to the Tip60 complex, but co-immunoprecipitated with PP2A Aα and PP2A Aβ, which means that it behaved like wild type (WT) ST for binding to these complexes. Thus, the point
mutants tested that no longer bind PP2A also have impaired binding to the Tip60 complex. It is possible that the residues which regulate the binding of MCPyV ST to PP2A are important for the structural integrity of ST. Alternatively, it is possible that PP2A acts as a bridge to Tip60 binding.

Tip60 complex mutants

To identify residues that mediate the binding between Tip60 complex and ST, without disrupting PP2A binding, we decided to map the interaction of ST with the Tip60 complex using alanine substitutions across loop 4. We hypothesized that since the interaction with the Tip60 complex is unique to MCPyV ST, the interaction most likely occurs in the previously discussed loop 4, which is between more conserved regions of the protein. Therefore, we began our mutagenesis in this region. Initially, Dr. Cheng performed deletion analysis along loop 4; however, all mutants failed to bind to PP2A, which suggests that the proteins were significantly altered and thus unsuitable for further analysis. Next, Dr. Cheng mutated 6-amino acid stretches to alanine to further map the binding. Both Dr. Cheng and I tested these mutants for interaction with MCPyV ST. The 83-88A (FPWEEY > 6A) ST mutant had undetectable binding to the Tip60 complex. When stably expressed in U2OS cells, the 83-88A mutant was unable to bind to the Tip60 complex but had slightly reduced binding to PP2A Aα and PP2A Aβ compared to wild type ST (Figure 2.5).

To further map the binding site for the Tip60 complex and to determine if I could further distinguish residues mediating binding to the Tip60 complex from PP2A binding, mutations scanning single or double amino acid residues within residues 83 to 88 were generated. The ST
mutants F83A, P84A, and Y88A retained binding to TRRAP, a Tip60 complex component, and therefore were not further characterized (Figure 2.5). Immunoprecipitation with Ab5 from cells expressing the W85A and E86A/E87A mutants did not have detectable binding to TRRAP but were able to co-precipitate PP2A Ala and PP2A Aβ (Figure 2.5). Later, Dr. Cheng created the W85S and the E86S/E87S ST mutants, which behaved similarly to the alanine mutants (Summarized in Table 2.1).

Dr. Cheng also created the 92S/93S double mutant to test for Tip60 binding. While this mutant still bound to PP2A and Tip60 in immunoprecipitation assays, it seemed to have increased expression levels and amplified the phenotype of WT ST in some assays performed in lab. The mechanism behind the mutant’s increased expression levels is yet unclear. Dr. Cheng also created a mutant with 86, 87, 92, and 93 mutated to serine (86/87/92/93S), which still lost binding to Tip60 but was expressed at higher levels than the E86S/E87S mutant.

Overall, we were able to identify three residues (W85, E86, and E87) in ST that regulate the interaction with the Tip60 complex but not PP2A.

Other mutants

Because we were thus far unable to identify a ST mutant that was unable to bind to PP2A yet retained binding to the Tip60 complex, I tested other ST mutants in our laboratory for PP2A binding. These mutants included the J domain point substitution mutant D44N, the triple point substitution 77-78-87A, and 7 point mutants of ST created by Christian Berrios. All mutants tested bound both PP2A and TRRAP (Summarized in Table 2.1). In all, we were unable to
identify a mutant that greatly reduced detection of binding to the PP2A complex while retaining binding to the Tip60 complex.
Figure 2.5: Identification of residues necessary for binding of ST to Tip60 complex proteins.

Lysates prepared from U2OS stable cell lines expressing the indicated ST construct or vector control were immunoprecipitated with Ab5, which recognizes ST and assayed by immunoblot for binding to TRRAP (Tip60 complex), PP2A Aα and PP2A Aβ. IgG control is immunoprecipitated from the ST cell line. * indicates expected size for light chain from immunoprecipitation; black arrow indicates expected size for ST. Vector, ST, and 83-88A cell lines are single cell cloned cell lines, accounting for the higher expression of ST, compared to the 83A, 84A, 85A, 86-87A, and 88A mutants. 83A, 84A, 85A, 86-87A, and 88A were expressed in U2OS stable cell lines selected with puromycin.
Discussion

Although MCPyV ST shares sequence homology with other polyomavirus STs, MCPyV ST has been reported to have some differences from the other STs in cellular binding partners. Unlike SV40 ST, our laboratory has observed that MCPyV ST uniquely binds to the Tip60 acetyltransferase complex in the Loop 4 region. In addition, MCPyV ST has recently been reported to bind to the ubiquitin ligase Fbw7, which prevents the Fbw7-mediated degradation of LT in the Loop 4 region [Kwun et al., 2013]. Thus, the Loop 4 region in MCPyV ST may mediate previously undiscovered functional interactions with cellular proteins.

By testing mutants, we were able map the binding of the Tip60 complex on ST and generate mutants that lost binding to Tip60 while retaining binding with PP2A. These residues were in the loop 4 region of ST, which was predicted to be structurally distinct from SV40 ST. Thus, we were able to map residues on ST that mediate the binding between ST and the Tip60 complex. Interestingly, the N terminal HA-FLAG double epitope tag (NTAP) ST construct also resulted in the disruption of binding of ST to the Tip60 protein complex. The mechanism for the alteration of binding is unclear at this point. The disruptive effect of the epitope tag on ST was somewhat unexpected but not surprising, given that the predicted structure for ST places the N-terminus towards the center of protein, rather than surface-exposed. This finding might be tested with other polyomavirus STs. The confounding effect of epitope tags may mean that experiments performed with tagged versions of ST may not uncover all functions of ST.

However, we were unable to characterize mutants that lost interaction with PP2A while retaining binding to the Tip60 complex. This could imply that the binding of ST to Tip60 is dependent on the ability of ST to interact with PP2A. It could also imply that the PP2A
holoenzyme is difficult to dissociate from a functional MCPyV ST. In either case, the lack of a clean PP2A mutant makes it more difficult to separate the functional implications of the binding of MCPyV ST with PP2A from the binding to Tip60.

Our data confirmed that MCPyV ST binds to both PP2A Aα and Aβ, like MPyV ST, which may have functional implications in both MCPyV infection and in MCC. The interaction of MCPyV ST with PP2A Aβ is notable because the MPyV ST interaction with PP2A Aβ is thought to account for some of the differences in function from SV40 ST. In particular, the MPyV ST interaction with the PP2A Aβ subunit is thought to alter cell signaling by PP2A, and may contribute to the MPyV ST-induced apoptosis of cells [Andrabi et al., 2011]. PP2A Aβ is involved in the negative regulation of the RalA GTPase [Sablina et al., 2007]. Mutations in PP2A Aβ are associated with some lung, colorectal, and breast cancers, as well as some melanomas [Calin et al., 2000; Tamaki et al., 2004; Wang et al., 1998]. Further investigation of the interaction of MCPyV ST with PP2A Aα and PP2A Aβ will be important for understanding functions of MCPyV ST in MCC.

These results lead us to the model that ST modulates binding to cellular proteins on two faces of the protein (Figure 2.6). An intact loop 4 is necessary for binding to the Tip60 complex and FBW7, and suggests that these cellular proteins might bind to this region of ST. In addition, we were unable to identify mutants of ST that lost binding to PP2A while retaining binding to components of the Tip60 complex. Thus, it is possible that the interaction with the Tip60 complex is related to the interaction with PP2A.
Figure 2.6: Model for ST binding. Different faces predicted from model of MCPyV ST modulate binding to various cellular proteins. ST binds to PP2A A subunit, leading to the displacement of the regulatory B subunit. ST also binds to the C subunit and is thought to potentially inhibit the catalytic activity, directly. These interactions are predicted to be mediated by residues in both the J domain and the unique domain [Cho et al., 2007]. ST has also been reported to bind to FBW7 on the opposite face of the PP2A interaction [Kwun et al., 2013], in the region that we call loop 4. We have also mapped the interaction with the Tip60 complex to loop 4.
Chapter III. Small T and DNA damage repair
Abstract

Many DNA viruses interfere with the cellular DNA damage response (DDR). In this chapter, we investigated DNA damage repair in cells that express Merkel Cell Polyomavirus (MCPyV) small T antigen (ST). The expression of ST changed the sensitivity of cells to DNA damage. In particular, cells expressing MCPyV ST experienced a large increase in sensitivity to ionizing radiation (IR) and etoposide. The difference in damage sensitivity to cisplatin, which causes platinum adducts that cannot be repaired by NHEJ, was not as large. In addition, the expression of ST caused the persistence of double strand DNA break (DSB) over time, as measured by neutral comet assay, indicating that DSB repair was perturbed. In mammalian cells, DSBs are primarily repaired by homologous recombination (HR) or nonhomologous end joining (NHEJ). To determine which repair pathways were affected by MCPyV ST, we used reporter assays to assess the repair by NHEJ and HR in cells. Using the Traffic Light Reporter assay, we observed that ST caused a shift in the proportion of breaks repaired by HR compared to NHEJ. Additional functional reporters showed that this shift was due to a decrease in NHEJ activity and an increase in HR in the presence of ST. These differences in repair pathway selection were not due to an alteration of cell cycle regulation by ST. In addition, we observed that there was a small difference in the kinetics of 53BP1 and RAD51 foci formation after damage in cells expressing ST. The presence of 53BP1 on a DSB will cause the break to be repaired by NHEJ, rather than HR. Together, these results show that MCPyV ST inhibits NHEJ. This phenotype is related to the increased persistence of DNA breaks in ST expressing cells and the no increase in sensitivity to cisplatin, which causes breaks that cannot be repaired by NHEJ.
Introduction

Many viruses interfere with the cellular DNA damage response as part of their life cycle (reviewed in Turnell and Grand, 2012). Initially, we began to examine if MCPyV ST has a role in the DNA damage response because we had seen that it interacts with the Tip60 chromatin remodeling complex, which has reported roles in DNA damage response and repair (reviewed in Sun et al., 2010). In addition, PP2A has been shown to be involved in the regulation of γH2AX signaling after DNA damage repair (Chowdhury et al., 2005).

Treatments for Merkel cell carcinoma (MCC), which express MCPyV proteins, include irradiation (IR) and chemotherapy that induces DNA damage, including etoposide and cisplatin (Nicolaidou et al., 2012). Therefore, we decided to examine the DNA damage response and repair pathways for breaks induced in cells that express ST.

Double strand breaks (DSB) can be more difficult to repair than other types of DNA damage in cells. In mammalian cells, DSBs are primarily repaired by homologous recombination (HR) or non-homologous end joining (NHEJ). HR and NHEJ are partially complementary in repair of DSB (Takata et al., 1998). Proper repair of DNA damage is essential for maintaining the integrity of the genome and preventing significant mutagenesis in cells.

HR is a method of repair of DSB with relatively high fidelity in mammalian cells. HR begins with end resection by the Mre11-Rad50-Nbs1 (MRN) complex and CtIP (Bressan et al., 1999; Limbo et al., 2007; Takeda et al., 2007; Williams et al., 2007). After end resection, single stranded DNA overhangs are initially coated by replication protein A (RPA), followed by RAD51 mediated strand invasion into the sister chromatid subsequently occurs (San Filippo et al., 2008). The association of BRCA1 and BRCA 2 with RAD51 is important for proficient
recombination and repair of breaks (Bhattacharya et al., 2000; Chen et al., 1998; Scully et al., 1997).

Repair by HR requires the presence of a DNA template with a high degree of homology to the site of the break. The sequence from the template is copied to repair the break, a process called gene conversion when the recipient DNA is gapped or broken. Most recombination events use information from the sister chromatid, but gene conversion is also possible from other homologous sequences within the genome at lower frequency (Johnson and Jasin, 2000; Richardson et al., 1998). Gene conversions can be classified as short tract gene conversion (STGC) or long tract gene conversion (LTGC), depending on the length of the DNA copied from the donor. In mammalian cells, most gene conversion events are short tract, with LTGC occurring as a result of abnormal HR (Chandramouly et al., 2013; Elliott et al., 1998).

NHEJ is the primary method for DSB repair for mammalian cells. However, NHEJ is thought to be more error prone than HR; the lack of a homologous template can lead to loss of nucleotides during the process of the damaged ends. In classic NHEJ, the DNA-PK holoenzyme, which includes the Ku70/80 heterodimer and the catalytically active kinase DNA-PKcs, binds to DNA ends at the DSB (West et al., 1998; Yaneva et al., 1997). Repair by NHEJ is carried out by a number of proteins, including the nuclease Artemis (DCLRE1C), XLF (NHEJ1), XRCC4, and DNA ligase IV (LIG4), which mediate processing and repair of the break (Lieber, 2008; Ma et al., 2002; Riballo et al., 2009; Roy et al., 2012).

The choice between HR and NHEJ is largely cell cycle dependent; NHEJ can occur during any stage of the cell cycle, while HR predominantly occurs in S-phase and G2 (Rothkamm et al., 2003). However, other factors contributing to the choice between these two
pathways are still being characterized. In part, it is thought that NHEJ and HR proteins compete at the site of DSB for repair; indeed, it has been shown that the presence of NHEJ proteins leads to an inhibition of HR \cite{Frank-Vaillant2002,Fukushima2001}. For example, the presence of Ku70/80 on DSB in ligase IV deficient cells can prevent HR from occurring on that break \cite{Adachi2001,Karanjawala2002}. In contrast, cells without XLF have a small increase of repairs by HR \cite{Fattah2014}. Many other proteins have been implicated in the commitment to a particular DSB repair pathway, 53BP1 being one of the most notable. The presence of 53BP1 on breaks is thought to protect the DSB from resection, and thus HR \cite{Bouwman2010,Bunting2010,Chapman2012}.

This chapter explores the effect of MCPyV ST on DNA damage repair pathways. Specifically, we determine if the expression of MCPyV ST could interfere with the repair of DNA by either HR or NHEJ. Altered DNA damage repair in the presence of ST has implications for both the initiation and the treatment of MCC.

**Contributions**

The reporter assays in this chapter were performed in collaboration with the laboratory of Dr. Ralph Scully (Beth Israel Deaconess Medical Center). Dr. Emilie Rass performed the NHEJ reporter assays shown in Figure 3.5B and Dr. Nick Willis performed the HR reporter assays shown in Figure 3.5C. All other assays discussed in this chapter were designed and performed by me.
**Materials and Methods**

**Cell culture and cloning.** Constructs were cloned into the Gateway vectors (Invitrogen) MSCV-N-Flag-HA-IRES-PURO (NTAP), MSCV-C-Flag-HA-IRES-PURO (CTAP) or the pLenti-CMV vector. Pooled U2OS cells and single cell cloned U2OS cells expressing vector or MCPyV ST were created and maintained as described in Chapter 2. For transient expression of ST in the I-SceI assays, ST constructs were expressed using a pLenti-CMV vector. For transient transfections, cells were transfected with Lipofectamine 2000 (Life technologies) following the manufacturer's protocol.

**Survival assays and DNA damage induction.** DSB were induced with ionizing radiation (IR) from a cesium-137 source in a Gammanell irradiator or by treating cells with etoposide or cisplatin.

For colorimetric assays, cells were counted and 50,000 cells were plated in each well of 6 well plates before being subjected to damage. After 14 days, cells were fixed and stained with crystal violet staining solution (Sigma) for 30 minutes and washed multiple times in water to remove excess solution. Plates were dried overnight. Crystal violet stain was re-dissolved in 500 \( \mu \)L of 10% acetic acid. Subsequently, the absorbance at 590 nm was measured in a Tecan plate reader. The surviving fraction was calculated by comparing the OD at each dose to the OD of control cells.

For clonogenic assays, 100, 500, or 1000 cells were counted and plated into each well of 6 well plates before being exposed to damage. After 13 days, cells were fixed and stained with crystal violet as in the colorimetric assays. The number of colonies on each plate was then
counted manually. The surviving fraction was calculated by comparing the number of colonies in each condition to the number of colonies in the unirradiated sample for each cell line assayed.

**Comet assay.** Cells were irradiated by cesium-137 source in a Gammacell irradiator. At the indicated times after irradiation, cells were counted and assayed using the manufacturer instructions with the comet assay kit (Trevigen). Comets were stained with Sybr gold (Life Technologies), imaged with a fluorescent microscope, and scored for the olive moment value using the OpenComet macro in ImageJ \cite{Gyori et al., 2014}.

**Reporter assays.** The traffic light reporter (TLR) assay was performed in single cell cloned U2OS cells stably expressing vector or ST and transduced with lentivirus expressing the TLR blue fluorescent protein (BFP) reporter \cite{Certo et al., 2011}. After 2 days, cells were transduced with the TLR I-SceI plasmid, to induce DSB. After 24 hours, cells were assayed by flow cytometry and gated for BFP+ cells.

For the HR and NHEJ assays, cells stably expressing the reporter construct were transiently co-transfected Lipofectamine 2000 (Invitrogen) and assayed by flow cytometry after 72 hours. The HR assay was performed in mouse ES cells as described in \cite{Nagaraju et al., 2006}, using a reporter construct that was modified to express RFP instead of blasticidin resistance. The NHEJ assays were performed as described in \cite{Xie et al., 2009}.

**Cell cycle profiling.** Cells were pulsed with 10μM Bromodeoxyuridine (BrdU) for 30 minutes. Cells were harvested, washed in PBS, and fixed in 70% EtOH and stored at -20° C. Cells were
then stained with anti-BrdU antibody (BD Biosciences) and PI, and assayed in the DFCI flow cytometry core facility.

**Immunofluorescence microscopy.** Cells were irradiated as described above. After the indicated time points, cells were fixed with 10% neutral-buffered formalin solution (Fisher). Coverslips were stained and slides were described as described in Chapter 2. Cells were scored for as positive or negative for foci, which was defined as >5 foci counted per nucleus.

**Results**

**Sensitivity to gamma irradiation**

Colorimetric analysis of cell survival post IR

To test if the presence of ST increased the sensitivity of cells to DNA damage, we performed damage sensitivity assays in U2OS cells. Attempts to perform this assay in IMR90 cells led to high variability between experiments. This was most likely due to the impaired ability of these cells to survive at lower plating density. In addition, MKL-1 and WaGa cells, MCC-derived cell lines, could not be adapted for this assay due to decreased cell viability at low plating concentrations and the higher margin of error in assessing the viability of these suspension cell lines. In addition, there is a lack of a good negative control for MCC cell lines, excepting shRNA against T antigens, but full knockdown is difficult to achieve due to low transfection efficiency in MKL-1 and WaGa. Thus, experiments testing the effect of ST on sensitivity to DNA damage were performed in U2OS cells.
Initially, U2OS stable cell lines expressing vector or untagged ST, without clonal selection, were plated at a density of 50,000 cells per well in a 6-well tissue culture plate. These cells were irradiated at 0, 4, and 6 Gy and assayed after 14 days for the surviving fraction. The relative density of cells was assessed by crystal violet staining and normalized to unirradiated control cells at 14 days to control for differences in cell growth and variation in plating between cell lines.

A smaller fraction of ST expressing cells survived after irradiation compared to vector expressing cells, indicating that cells expressing ST were more sensitive to irradiation than vector control cells (Figure 3.1A). The differences were not statistically significant.

Clonogenic DNA damage survival assays

One issue with the colorimetric survival assay is the high experimental variability due to limitations of the crystal violet staining. Despite the fact that cells containing ST consistently had lower survival than vector cells in the colorimetric assay, I was unable to get statistically significant results. Therefore, I switched to a clonogenic survival assay.

The clonogenic survival assay assesses the cells in a more discrete manner. Instead of pooling the cells and testing for overall cell density, the clonogenic survival assay involves plating cells at lower densities (100, 500, and 2000 cells per well in 6-well plate) and counting the number of colonies that survive. This assay, therefore, excludes cells that divide only a few times before undergoing senescence or apoptosis.
For the clonogenic survival assay, I also used single cell clones that expressed vector, ST, or 83-88A ST, which is a mutant of ST that disrupts Tip60 binding. Generation and characterization of these cell lines and characterization of the mutant is discussed in Chapter 2. For each of the three biological replicates, two different vector single cell clones, three different ST expressing single cell clones, and two different 83-88A expressing single cell clones were used, with two technical replicates of each clone per experiment. After 13 days, colonies were stained with crystal violet and counted. The number of surviving colonies was normalized to the unirradiated control for each cell line. The surviving fraction was analyzed.

In this assay, cells expressing ST were significantly more sensitive to irradiation than the vector control (Figure 3.2A). The 83-88A ST had similar survival in response to ionizing radiation to wild type (WT) ST.
Figure 3.1: ST expressing cells are more sensitive to irradiation and etoposide. In the colorimetric survival assay, pooled U2OS cell lines expressing vector or ST were subjected to increasing doses of (A) irradiation or (B) etoposide. After 14 days following treatment, cells were fixed and stained with crystal violet. Subsequently, the amount of crystal violet was measured in a spectrophotometer after re-dissolving the crystal violet in 10% acetic acid. The surviving fraction was calculated by comparing the OD at each dose by the OD of untreated cells. Error bars show standard error of mean across three biological replicates. Each biological replicate is the average of two technical replicates. Differences between vector and ST were not significant (p > 0.05) when assayed with two-sided Student’s t-test.
**Sensitivity to Etoposide**

We also examined sensitivity to DNA damage induced by etoposide using the colorimetric survival assay. Etoposide is a topoisomerase inhibitor that causes DSB \(^{(Wozniak and Ross, 1983)}\) and was used as a different method for damage induction. U2OS cells without clonal selection were plated at a density of 50,000 cells per well in a 6-well tissue culture plate and treated with 0, 4, and 20 µM etoposide to induce damage. After 14 days, cells were harvested with crystal violet and relative cell density was measured by spectrophotometry. The relative cell density was normalized to control cells that were treated with DMSO.

Cells expressing ST were more sensitive to DNA damage from etoposide treatment, but the differences were not statistically significant (Figure 3.1B). The sensitivity to etoposide was not tested in the clonogenic survival assay.

**Sensitivity to Cisplatin**

Cisplatin causes platinum adducts that are recognized as DSB \(^{(Jamieson and Lippard, 1999)}\). Due to the nature of the platinum adducts, cisplatin adducts cannot be repaired by NHEJ \(^{(Boeckman et al., 2005)}\). Thus, if ST affects NHEJ but not HR, we would expect that ST would not show increased sensitivity to cisplatin compared to vector.

For this assay, 100, 500, and 1000 cells were plated in each well of a 6-well plate. Cells were plated into media that contained cisplatin. For each biological replicate, two vector single cell clones and three ST expressing single cell clones were used with two technical replicates of each sample. After 13 days, colonies were stained with crystal violet and counted. The number of
surviving colonies was normalized to the negative control for each cell line and the surviving fraction was analyzed.

When the cells were assessed by clonogenic survival assay, ST cells were slightly more sensitive to cisplatin (Figure 3.2B). This difference was not statistically significant despite the low variability in the assay.
Figure 3.2: ST expressing cells are more sensitive to irradiation but not cisplatin. (A) Single cell cloned U2OS stable cell lines expressing vector, ST, or 83-88A ST were counted, plated, and subjected to irradiation. After 13 days, cells were fixed and stained with crystal violet. The number of colonies per well was counted and the surviving fraction was calculated by comparing the number to untreated controls. Significance was determined using two-sided Student’s t-test. p < 0.05 for vector and ST at 4 Gy; and for vector and 83-88A ST at 2, 4, and 6 Gy. p < 0.005 for vector and ST at 2 and 6 Gy. (B) Single cell cloned U2OS stable cell lines expressing vector or ST were treated for cisplatin and assayed for surviving fraction in the clonogenic survival assay. Differences between vector and ST survival were not significant (p > 0.05) when measured using two-sided Student’s t-test. For both assays, error bars show standard error of mean across three biological replicates. Each biological replicate is the average of 2 vector single cell clones and 3 ST single cell clones (described in chapter 2), with two technical replicates for each cell line.
Double stranded DNA break repair

To determine if MCPyV ST had an effect on the rate of repair of DSB, I treated cells with a large dose of ionizing radiation and measured the repair of DSB over time with a neutral comet assay. Comet assays are a single cell gel electrophoresis assay with lysis of cells in situ within agarose on slides. Neutral comet assays primarily detect DSB in DNA, whereas alkaline comet assays are better suited for detecting single stranded breaks. The amount of fragmented DNA in the “tail” of the comet can be compared to the amount of DNA that is relatively intact in the “head” of the comet. The quantitative measure of this value is called the “olive moment”, which is expressed as the product of the fraction of total DNA in the tail and the tail length. Due to the nature of the assay, the amount of damage induced results in a very large number of DSB.

Single cell clones of U2OS cells stably expressing vector or ST, generated and described in Chapter 2, were irradiated at 40 Gy or left untreated and harvested at various time points after IR. Comets were scored for olive moment. For each biological replicate, one control vector cell line and one ST cell line was tested. Three different ST clones were tested independently. Cells expressing ST had a slower rate of repair of dsDNA breaks compared to the vector control cells (Figure 3.3), indicating that ST expressing cells were slower or unable to repair the majority of the breaks.
Figure 3.3: DSB persistence in ST cells after IR. Single cell cloned U2OS stable cell lines expressing vector or ST were irradiated with 40 Gy, stained with Sybr gold, and assayed at the times indicated by neutral comet assay. Comets were scored for olive moment and values were expressed in comparison to the olive moment at 1 hour post IR. The 0 h time point indicates the basal damage level of cells before irradiation. (A) Representative comets as imaged by immunofluorescence microscopy at the indicated time points after IR. (B) Relative olive moment of comets. Significance was determined using 2 way ANOVA analysis (p < 0.05). Error bars show standard error of mean across three biological replicates. Each biological replicate is performed with vector single cell clone 3 and a different ST single cell clone (described in Chapter 2), with two technical replicates for each cell line.
Perturbation of DNA damage repair pathways

Since we observed a defect in dsDNA damage repair, we decided to examine how the major dsDNA damage repair pathways, HR and NHEJ, were affected by the presence of ST. Initially, to gain a better understanding of the perturbations caused by ST, we assayed cells with the Traffic Light Reporter system (TLR) (Certo et al., 2011). The TLR system uses a specific endonuclease, I-SceI, which cuts a unique site in a reporter construct. If the I-SceI digested DNA is repaired by NHEJ, the repaired construct expresses mCherry because the GFP gene is translated out of frame while the mCherry sequences are rendered in frame. If the DSB induced by I-SceI is repaired by HR, then the reporter will express GFP because the full GFP gene will be reconstituted. By determining the fraction of cells expressing mCherry or GFP, we can determine the relative ratio of repair by HR or NHEJ (Figure 3.4A).

For this experiment, cells were plated in a 6 well plate approximately 1 day before transduction. Cells were then transduced with the TLR reporter lentivirus, which expresses a BFP marker, for 48 hours. Each well was then split into multiple 6-well plates and transduced with the TLR lentivirus containing the I-SceI endonuclease to induce DSB. After 48h, cells were assayed by flow cytometry, gating on BFP positive cells to ensure that assayed cells contain the I-SceI site, and checking for the numbers of cells that expressed mCherry or GFP. The TLR assay revealed that in the presence of ST, there was a strong shift in the ratio of HR:NHEJ with relatively higher amounts of HR (GFP) than NHEJ (mCherry). This indicates that ST expressing cells strongly favor HR over NHEJ (Figure 3.5A). This could indicate that there was a strong preference for HR in the ST expressing cells or that ST inhibits NHEJ. With the TLR assay, however, it is not possible to distinguish between these two possibilities. The TLR system
allowed us hypothesize that either NHEJ was inhibited or that HR was increased in cells expressing ST. Therefore, we decided to further pursue the differences in repair by HR and NHEJ in the presence of ST.

In collaboration with Dr. Emilie Rass and Dr. Nick Willis in Dr. Ralph Scully’s laboratory (Beth Israel Deaconess Medical Center), we next assayed the abilities of ST to alter repair by HR or NHEJ. First, the effect of ST on NHEJ was assayed in a U2OS based NHEJ reporter system (Xie et al., 2009). In this assay, cells contain a single copy of a construct that contains an I-SceI site are transiently co-transfected with I-SceI expression construct. If the I-SceI break is repaired by NHEJ, cells express GFP. If the break is repaired by a different mechanism, no GFP is expressed (Figure 3.4B). Cells are typically assessed for GFP expression by flow cytometry 48 hours after the induction of the break.

In the NHEJ assay, we tested MCPyV ST, and the ST mutants E86S/E87S ST, 92/93S ST, and 86/87/92/93S ST, which are discussed in detail in chapter 2. The E86S/E87S ST and 86/87/92/93S ST are unable to bind to the Tip60 complex (Summarized in Table 2.1). In addition, mutating residues 92 and 93 led to an increase in the overall levels of ST in transient transfections, which had resulted in increased ST phenotypes in other assays in our lab. We observed that transient transfection of WT ST led to a decrease of approximately 70% in NHEJ, indicating that the presence of ST inhibited NHEJ (Figure 3.5B). The E86S/E87S and the 86/87/92/93S mutants had 68% decrease and 58% decrease in NHEJ respectively, which is similar to the result we saw with WT ST. In addition, the 92/93S mutant behaved similarly to WT ST, with a 74% inhibition of NHEJ.
Figure 3.4: Diagrams of I-SceI reporter assays. Schematics showing the reporter systems for the
(A) TLR assay [Certo et al., 2011]. (B) NHEJ assay [Xie et al., 2009]. (C) HR assay [Nagaraju et
al., 2006].
Figure 3.5: HR and NHEJ activity is altered in the presence of ST. (A) The ratio of breaks repaired by HR over NHEJ as assessed by TLR assay. The TLR assay was performed in U2OS single cell cloned cell lines that stably express vector or ST. Significance was determined using two-sided Student’s t-test. Differences between vector and ST were not statistically significant (p > 0.05). Each of two biological replicates is the average of results for 2 vector single cell clones and 3 ST single cell clones (described in chapter 2). (B) NHEJ assay in U2OS cells. The NHEJ assay was performed with transient transfection of vector or ST into stable cell lines expressing the reporter construct. Significance was determined using two-sided Student’s t-test (* p < 0.05).
Figure 3.5: HR and NHEJ activity is altered in the presence of ST (Continued).

Differences between ST and ST mutants were not significant (p > 0.05). (C) HR assay in mouse ES cells. The HR assay was performed with transient expression of vector or ST into stable cell lines expressing the reporter construct. Significance was determined using two-sided Student’s t-test. Differences between vector and ST were not significant (p > 0.05) across four biological replicates. For all assays, error bars show standard error of mean.
Next, we tested if repair by HR was altered in the presence of ST. The effect of ST on HR was assayed in a mouse ES based HR system [Nagaraju et al., 2006]. In this assay, mouse ES cells stably express a construct that contains the I-SceI site. I-SceI is co-transfected with vector control or ST. If the I-SceI-induced break is repaired by short tract gene conversion (STGC), the construct is repaired in a way that GFP is expressed. However, if the repair is by long tract gene conversion (LTGC), the cell will express both GFP and RFP. In this system, if the repair is by NHEJ, neither GFP nor RFP is expressed (Figure 3.4C). Similarly to the NHEJ assay, cells were assessed by flow cytometry for GFP or RFP expression after induction of the break by I-SceI.

In this assay, we observed that transient transfection of ST led to a measurable increase in overall HR with no change in the preference STGC or LTGC, which are subtypes of HR (Figure 3.5C). The number of STGC events (GFP+, RFP-) measured increased by 61% compared to vector and the number of LTGC events (GFP+, RFP+) measured increased by 61% compared to vector, as well. These results indicate that ST increases the frequency of HR without altering the type of HR preferred. The larger variation observed with this assay was due to the variations in the plasmid preps used across experiments; however, in each of the four biological replicates performed, the measurable difference in HR was consistently higher in the ST expressing cells.

As controls for the repair assays, we checked if the effect of ST due to an increase in S/G2 in ST expressing cells, since the choice for repairing breaks by NHEJ or HR is largely dependent on the cell cycle. In cells that stably express ST or after 72h transient transfection, I observed that there was no significant difference in the cell cycle of ST expressing cells (Figure 3.6). Thus, the alteration in NHEJ and HR is not a byproduct of altered cell cycle regulation.
Through these assays, we observed the presence of ST increased the preference of HR over NHEJ, resulting in an absolute increase in HR and decrease in NHEJ. This phenotype indicates that the expression of ST leads to a defect in repair of DSB by NHEJ.
Figure 3.6: Cell cycle distribution of cells is not significantly altered by ST expression. To assess cell cycle distribution, cells were pulsed with 10µM BrdU for 30 minutes and stained for BrdU and PI. Samples were analyzed by flow cytometry. (A) The cell cycle distribution of single cell cloned U2OS cells stably expressing vector or ST. Each of three biological replicates was performed with one vector subclone and one ST subclone (described in chapter 2). Differences between vector and ST were not significant (p > 0.05) when measured using two-sided Student’s t-test. (B) Cell cycle distribution of U2OS cells transiently transfected with Lipofectamine 2000 with vector or ST expression constructs. Cells were harvested 72 hours after transfection. Differences between vector and ST were not significant (p > 0.05) when measured using two-sided Student’s t-test.
Localization of 53BP1 and RAD51 on breaks

One mediator of the choice between the NHEJ and HR repair pathways is the amount of 53BP1 at sites of damage. The presence of 53BP1 on breaks is thought to promote NHEJ while specifically preventing HR \cite{Bouwman2010,Bunting2010,Chapman2012}. Since we observed a difference in NHEJ in the presence of ST, I tested whether I could detect a difference in the appearance or number of 53BP1 foci in the presence or absence of ST. RAD51 is used in this assay as a marker for breaks that will are repaired by HR.

A time course was performed to examine the kinetics of RAD51 and 53BP1 foci as detected by immunofluorescence after cells were treated with ionizing radiation to induce DNA damage. Single cell cloned cells expressing vector or ST were irradiated with 5 Gy, harvested and fixed at intervals over 42 hours, and stained with antibody against RAD51. In this time course, ST expressing cells had more RAD51 positive cells compared to vector control cells at some time points, but there was little distinction in the overall kinetics or intensity (Figure 3.7). This experiment was repeated over a shorter time course. Single cell cloned cells expressing vector or ST were irradiated with 5 Gy, harvested and fixed at 1 or 6 hours after irradiation, and stained with antibody against RAD51 and 53BP1. ST expressing cells contained more RAD51 foci than vector control cells 1h post IR (Figure 3.8). In addition, there was a small decrease in the number of cells with 53BP1 foci at 6h, although this difference was not statistically significant.
Figure 3.7: RAD51 kinetics is similar in vector and ST expressing cells. U2OS single cell cloned cell lines that stably express vector or ST were grown on cover slips, irradiated with 5 Gy and harvested at various time points after IR. Coverslips were stained with primary antibody against Rad51, followed by secondary antibody conjugated to Alexa-488 and DAPI. Rad51 foci were visualized using immunofluorescence microscopy. (A) Example of images used for scoring. Cells with more than 5 foci per nuclei were scored as positive. Scoring was performed manually.
Figure 3.7: RAD51 kinetics is similar in vector and ST expressing cells (Continued).

(B) Quantification of Rad51 positive cells over time after IR. Differences between vector and ST were not significant (p > 0.05) when measured using two-sided Student’s t-test. Each of three biological replicates was performed with one vector subclone and one ST subclone (described in chapter 2).
Figure 3.8: ST may alter RAD51 but not 53BP1 kinetics after IR. U2OS single cell cloned cell lines that stably express vector or ST were grown on cover slips, irradiated with 5 Gy and harvested at various time points after irradiation. Coverslips were stained with primary antibody against Rad51 and 53BP1, followed by secondary antibody conjugated to Alexa-488 or cy3 and DAPI. Rad51 foci and 53BP1 foci were visualized using immunofluorescence microscopy. Differences between vector and ST were not significant (p > 0.05) when measured using two-sided Student’s t-test. Each of three biological replicates was performed with one vector subclone and one ST subclone (described in chapter 2).
Tip60 localization after IR

Because ST binds to the Tip60 complex, I wanted to directly test the hypothesis that ST affects the role of the Tip60 complex in cells undergoing DNA damage. If the Tip60 complex is altered after irradiation, this could imply a role for the Tip60 complex in the ST-mediated increase in DNA damage sensitivity.

Cells were fractionated in 3 steps to obtain a cytoplasmic, nuclear, and chromatin enriched fractions. These fractions were assessed by immunoblot to determine if there was a difference in the amount of Tip60-related proteins in the chromatin-enriched fraction. Tubulin, lamin, and H2AX were used as controls for the fractionation. Tubulin was observed in the cytoplasmic fraction, lamin was observed in both the nuclear and chromatin enriched fractions, and H2AX was observed only in the chromatin-enriched fraction (Figure 3.9).

ST expressing cells have increased levels of Tip60 complex in the chromatin-enriched fraction both with and without IR (Figure 3.9). This indicates ST may affect the localization of the Tip60 complex. The localization of Tip60 complex proteins did not change after exogenous damage by IR. In addition, ST is present in all of the fractions, confirming our observation in Chapter 2 that it is in both cytoplasmic and nuclear compartments.
Figure 3.9: Tip60 complex components are enriched in the chromatin fraction of ST cells. Pooled U2OS cells that stably express vector or ST were lysed sequentially cytoplasmic buffer (CSK), EBC buffer, and finally in RIPA buffer. Lysates were assessed by immunoblot for Tip60 complex components. Expression of lamin, tubulin, and total H2AX was assessed as a control for the fractionation.
Discussion

In this chapter, we observed that MCPyV ST inhibits the repair of DSB by NHEJ and increases repair by HR. In addition, we observed the persistence of unresolved DSB after DNA damage and an increased sensitivity to DNA damage by IR that could be explained by the ST inhibitory effect on NHEJ. Understanding the inhibition of NHEJ by ST could reveal insights into how the process of NHEJ unfolds in mammalian cells.

Compared to WT ST, the Tip60 complex binding-defective E86S/E87S mutant and the 86/87/92/93S mutant had slightly less of an inhibition of NHEJ than what we saw with WT ST. However, the difference between WT ST and the two Tip60 complex binding mutants was not significant in this assay. In addition, tests with Tip60 complex binding defective mutant 83-88A ST in the clonogenic DNA sensitivity assay also showed no difference with wild type ST. Thus, it appears that the interaction of ST and the Tip60 complex did not contribute to the increased sensitivity to DNA damage. It is yet unclear if the effect of ST on NHEJ is caused by the effect of ST on PP2A function. Thus, further work is needed to identify the cellular mediators of the inhibition of NHEJ by ST.

Other DNA viruses have been reported to block parts of the DNA damage repair pathways or alter the preference for one pathway over another. One well-characterized example of this phenomenon is with adenovirus. During the course of infection, adenovirus causes degradation and mislocalization of the MRN complex to prevent concatemerization of its genome \cite{Boyer1999,Stracker2002,Weiden1994}. The production of multimeric genomes can be rather problematic for the viral life cycle. Adenovirus causes the degradation of ligase IV in an E4 34k/E1b 55k complex dependent manner \cite{Baker2007}.
The genomic instability caused by blocking of DNA response pathways could partially explain the “hit-and-run” hypothesis: where the genomic mutations caused by altered DNA repair pathways lead to cancer (Nevels et al., 2001; Stracker et al., 2002). JC polyomavirus has also been reported to cause the inhibition of DSB repair pathways by modulating the levels of Ku70/Ku80, which are part of the DNA-PK complex (Darbinyan et al., 2004). Therefore, the alteration of DNA damage repair may have roles in the MCPyV life cycle.

One possible result of ST alterations of repair pathways could be a lower rate of random integration of the viral genome as observed in Merkel cell carcinomas (MCC). In MCC, the virus is clonally integrated in both the primary tumor and metastatic tumors (Feng et al., 2008; Laude et al., 2010). Mutations in LT that eliminate the ability of the viral genome to replicate seem to be necessary for the formation of MCC (Shuda et al., 2008). MCPyV has no proteins that can allow excision from the host genome, and thus integration is a dead end for viral replication and propagation. Thus, blocking spontaneous integration could be advantageous for viral infection and replication.

Taken together, these results show that ST can inhibit DNA damage repair by NHEJ but does not inhibit repair by HR (Figure 3.10). This decrease in repair may result in the persistence of DNA damage breaks and the decreased cell survival after damage.
Figure 3.10: Summary of ST role in DNA damage sensitivity. Expression of ST inhibits NHEJ in cells, which can lead to decreased total DSB repair. The increase in DNA damage sensitivity with ST can be attributed to the effect of ST on DNA damage repair by NHEJ.
Chapter IV. Small T and DNA damage signaling
Abstract

In chapter 3, we observed that the expression of MCPyV ST resulted in an increase in DNA damage sensitivity and a decrease in DNA damage repair by NHEJ. We wanted to determine if ST also perturbed the signaling that regulates DNA damage detection or repair. We investigated the activation of two protein kinases, ATM and DNA-PK. We observed that after treatment of cells with ionizing radiation, the activating phosphorylation of ATM at serine 1981 was similar in cells expressing vector and ST. Chk-2 phosphorylation, which is mediated by ATM, was also similar to control cells. The DNA-PK kinase is regulated by a number of phosphorylation events on the catalytic subunit, DNA-PKcs. After exposure to γ-irradiation, DNA-PKcs was phosphorylated to a lesser degree on S2056 in ST expressing cells compared to vector control. Finally, we investigated the perturbations of ST on the p53/p21 downstream response pathway, which regulates the choice between growth arrest and apoptosis. While IR induced normal p53 phosphorylation and increased p53 stability, p21 levels were lower in ST expressing cells. These results together show that signaling related to NHEJ is altered when ST is expressed, which is consistent with the inhibition of NHEJ by ST discussed in the previous chapter.
**Introduction**

In chapter 3, we explored the inhibition of repair pathways by MCPyV ST after exogenous sources of double strand DNA (dsDNA) damage. We observed that MCPyV ST inhibited double strand DNA break (DSB) repair by non-homologous end joining (NHEJ). In this chapter, we examine the role of MCPyV ST in cellular DNA damage checkpoint signaling pathways to further understand how ST disrupts the DNA damage response.

The DNA damage signaling pathways are essential for the repair of DSB. The generation of a DSB leads to the rapid recruitment and activation of one of three sensor proteins, ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related protein (ATR) and DNA-dependent protein kinase (DNA-PK), which form a group called the phosphatidylinositol 3-kinase-related kinase (PIKK) family. The activity of ATM, ATR and DNA-PK leads to the recruitment of mediators of DNA damage repair and activation of the DNA damage checkpoint (reviewed in (Durocher and Jackson, 2001)). In mammalian cells, signaling for DSB repair is largely regulated by the kinases ATM and DNA-PK, which have roles in activating effector molecules in repair and in recruiting repair molecules to the site of the breaks.

The activation of ATM, a serine/threonine kinase, is an important step in the DNA damage response. ATM is activated in part after interaction with the MRE11-RAD50-NBS1 (MRN) complex and in response to changes in chromatin structure around the site of the break (Bakkenist and Kastan, 2003; Uziel et al., 2003). After DNA damage, inactive ATM dimers are activated through dissociation and trans-autophosphorylated at serine 1981 (Bakkenist and Kastan, 2003). In unirradiated cells, phosphorylation at S1981 is negatively regulated by interaction with PP2A; this interaction is disrupted by IR (Goodarzi et al., 2004). In addition to
the phosphorylation at S1981, ATM ribosylation by PARP1 and acetylation at K3016 by Tip60 is also required for activation after DNA damage (Aguilar-Quesada et al., 2007; Sun et al., 2007; Xu et al., 2011).

The DNA-PK kinase is made up of the catalytic subunit DNA-PKcs together with Ku70 and Ku80. Regulation of DNA-PK activity involves phosphorylation of multiple residues on its subunits. DNA-PKcs is phosphorylated in multiple regions that are thought to affect different DNA-PKcs functions. DNA-PKcs is phosphorylated at two major clusters, the ABCDE cluster (T2609, S2612, T2620, S2624, T2638, T2647) and the PQR cluster (S2023, S2029, S2041, S2053, S2056) (Chan et al., 2002; Cui et al., 2005; Ding et al., 2003) (Figure 4.1). Auto phosphorylation at the ABCDE sites causes a rearrangement of the DNA-PK complex that allows for access to the break and disassembly from the DSB sites (Chan and Lees-Miller, 1996; Ding et al., 2003; Reddy et al., 2004). It has also been reported that DNA-PKcs phosphorylation in the ABCDE cluster are important for full NHEJ activity, although they are non-essential (Povirk et al., 2007). Initially, the ABCDE and PQR clusters were thought to reciprocally regulate end-processing functions in NHEJ, with phosphorylation at ABCDE promoting end processing and phosphorylation at PQR decreasing end processing (Cui et al., 2005). However, it has been shown more recently using a different reporter system that mutating the ABCDE sites to alanines also promotes HR over NHEJ, indicating that the regulation of DNA-PK activity is more complex than initially reported (Neal et al., 2011). In addition to the major clusters, DNA-PKcs has been reported to phosphorylate T3950 in response to DNA damage and mutation the T3950A mutation caused increased DNA damage sensitivity (Douglas et al., 2007). These phosphorylation sites are summarized in Figure 4.1.
Figure 4.1: Diagram of DNA-PKcs phosphorylation sites. Asterisks indicate the phosphorylation sites discussed here. The kinase domain is indicated by the grey box. Figure adapted from Neal et al., 2014.
Activation of ATM and DNA-PK activity can cause amplification of DNA damage signaling with the phosphorylation of H2AX. Upon DNA damage, histone H2AX surrounding the site of the break becomes phosphorylated at S139 [Rogakou et al., 1998]. This phosphorylation, called \( \gamma \)H2AX, form foci that are readily visible by immunofluorescence staining within minutes after damage and the number of foci is related to the number of breaks formed [Paull et al., 2000; Rogakou et al., 1999]. The phosphorylation of H2AX was initially thought to be regulated solely by ATM [Burma et al., 2001]. However, it has been reported that ATM, DNA-PK and ATR function redundantly in this process [Stiff et al., 2004]. Though DNA-PK and ATM are readily activated by DSBs, ATR has a much higher affinity towards RPA coated single stranded breaks and is most potently activated by stalled replication forks and end resected DSBs [Ward and Chen, 2001]. Inhibition of \( \gamma \)H2AX formation results in a decrease in recruitment of the DNA damage proteins RAD51 and BRCA1 [Paull et al., 2000], indicating that \( \gamma \)H2AX is important for the recruitment of downstream mediators of the DNA damage response.

Interestingly, signaling and effector proteins including the MRN complex, 53BP1, and BRCA1 are recruited at low levels to sites of DNA damage in H2AX null cells but do not form foci [Bouquet et al., 2006; Celeste et al., 2003]. This indicates that \( \gamma \)H2AX may be not be responsible for the initial recruitment of these factors but are important in concentrating the factors on the sites of the breaks. H2AX null cells have increased sensitivity to IR [Celeste et al., 2003]. Therefore, \( \gamma \)H2AX is an essential part of the signaling cascade after DNA damage.

ATM activity is required for the downstream phosphorylation of regulators of both DNA damage repair and DNA damage checkpoint pathways. ATM phosphorylates both CHK1 and CHK2, upon DNA damage [Gatei et al., 2003; Matsuoka et al., 1998; Matsuoka et al., 2000].
CHK2 activity is necessary for regulating DNA checkpoints after damage and contributes to the activation of p53 with the phosphorylation at serine 20 [Hirao et al., 2000]. ATM also phosphorylates p53 at S15, leading to the accumulation of p53 and allowing p53 to transactivate downstream target genes [Banin et al., 1998; Canman et al., 1998]. p53 activation leads to the transcription of multiple genes, including p21, which in turn regulates cell cycle progression [el-Deiry et al., 1993]. In addition, ATM phosphorylates MDM2, BRCA1, and NBS1, which are involved in homologous repair [Khanna and Jackson, 2001].

DNA-PKcs has been reported to phosphorylate a number of proteins involved in NHEJ in vitro. Notably, although DNA-PKcs phosphorylates Ku70 on serine 6, and Ku80 on serine 577, serine 580, threonine 715, these residues do not seem to be important for repair by NHEJ [Chan et al., 1999; Douglas et al., 2005]. Also, the DNA-PK-mediated phosphorylation events on XRCC4 are not required for NHEJ [Yu et al., 2003]. Thus, only phosphorylation sites on DNA-PKcs itself have been shown to be important for normal NHEJ.

However, the ATM and DNA-PK pathways are not fully independent of each other in the context of DSB repair. For example, blocking the phosphorylation of the PQR cluster of DNA-PKcs promotes HR [Cui et al., 2005] and DNA-PKcs that is enzymatically active and promotes NHEJ represses HR [Neal et al., 2011]. ATM has been reported phosphorylate T2609 on DNA-PKcs after damage [Chen et al., 2007a]. In addition, ATM and DNA-PK have been shown to have redundant functions in phosphorylation of proteins such as γH2AX [Stiff et al., 2004].

In this chapter, we examine the effects of MCPyV ST on signaling pathways that are relevant for DSB repair. Specifically, we examine signaling pathways related to ATM and DNA-PK. In addition, we examine general markers of DNA damage, including γH2AX. Understanding
perturbations of DNA damage signaling events by MCPyV ST can shed light on the molecular mechanism for the increase in sensitivity to damage.

Contributions

All assays discussed in this chapter were designed and performed by me.

Materials and Methods

Cell culture and cloning. U2OS stable cell lines and single cell cloned U2OS cells expressing vector or MCPyV ST were created and maintained as described in Chapter 2.

DNA damage. Double strand DNA breaks were induced with ionizing radiation from a Cesium-137 source. The Gammacell 40 Exactor or the Gammacell 3000 irradiator are part of the DFCI core.

Immunoblots. Cells were harvested and washed in 1x PBS and lysed in EBC buffer (50 mM Tris HCl pH8, 150 mM NaCl, 0.5% NP40, 0.5mM EDTA pH8) containing phosphatase and protease inhibitors (Calbiochem). Lysates were collected by centrifugation at maximum speeds. Lysates were combined with 1x Laemmli buffer and run on 4-20% TGX gradient gels (Bio-Rad) and transferred onto nitrocellulose membranes.

For blots assessed with Odyssey Licor, the proteins were assayed using Licor membranes, blocking buffer, and secondary antibodies using the manufacturer’s instructions. Quantification
of signal intensity in immunoblots was performed with Image Studio Light (Licor), using the manufacturer’s instructions.

Immunoblotting was performed with antibodies to Ku70 (Abcam), Ku80 (Abcam), phospho DNA-PKcs S2056 (Abcam), total DNA-PKcs (Santa Cruz), DNA Ligase IV (Santa Cruz), phospho ATM 1981 (Cell Signaling), total ATM (Genetex), total p53 (Santa Cruz), phospho p53 S15 (Cell Signaling), p21 (Cell Signaling), phospho CHK2 T68 (cell signaling), α-tubulin (Genetex), and ST (described in Cheng et al., 2013; Rodig et al., 2012).

Immunofluorescence microscopy. After damage by irradiation, slides were prepared as described in Chapter 2. The number of foci per cell was calculated using macros in ImageJ.

Results

Activation of mediators of DNA damage repair

Since we had previously observed that ST leads to a significant decrease in repair by NHEJ, we decided to examine the molecular events upstream of repair. Specifically, I examined the phosphorylation of ATM and the phosphorylation of DNA-PKcs after gamma irradiation in single cell cloned cell lines expressing vector or MCPyV ST. Initially, we examined the activation of ATM at serine 1981. Phosphorylation of this residue is important for the activation of ATM (Bakkenist and Kastan, 2003; So et al., 2009). Both ST expressing and vector control cells induced similar levels of phosphorylated ATM and its downstream effector kinase CHK2.
after IR when assayed by immunoblot (Figure 4.2). This result was consistent across three biological replicates.

Next, we tested if there was a difference in the activation of DNA-PK through the phosphorylation of the catalytic subunit, DNA-PKcs. The single cell cloned cell lines that express vector or ST (described in Chapter 2) were irradiated with 5 Gy and harvested at 5, 15, and 30 minutes after damage to assess the levels and kinetics of DNA-PKcs phosphorylation at S2056, which is in the well characterized PQR cluster. When samples were assayed by immunoblot, I observed a reproducible decrease in the total amount of phospho-DNA-PKcs at residue S2056 (Figure 4.3A). With the Licor, the signal at 0, 5, 15, and 30 minutes after irradiation was quantified. The signal intensity was normalized to the total DNA-PKcs signal. The signal at phospho-DNA-PKcs S2056 was approximately 60% lower in ST expressing cells (Figure 4.3B). In addition, since we performed these experiments in stable cell lines that had been selected for high levels of ST, I also checked steady state levels of four other mediators of NHEJ: XLF, Ligase IV, Ku70 and Ku80. The levels of these four proteins were similar in vector and ST expressing cells, indicating that the stable expression of ST did not alter their overall protein levels.

Given the apparent decreased phosphorylation of DNA-PKcs S2056 after IR in ST expressing cells, I evaluated the intensity and total number of phospho-DNA-PKcs S2056 foci after irradiation by immunofluorescence microscopy. I stained foci with phospho-S2056 antibody and quantified the number of foci with ImageJ. Representative images of phospho-DNA-PKcs foci are shown in Figure 4.3C. In addition, I observed a 30% decrease in the number
of foci (Figure 4.3D). Therefore, the presence of ST decreases an important phosphorylation in
the PQR cluster region of DNA-PKcs.
Figure 4.2: ATM signaling is not inhibited in ST expressing cells. Single cell cloned U2OS stable cell lines expressing vector or ST were irradiated with 5 Gy and harvested at the indicated times. Levels of pS1981 ATM, total ATM, pT69 CHK2, ST, and tubulin at each time point were determined by immunoblot. Blot is representative of three independent experiments. Each biological replicate is performed with vector single cell clone 3 and a different ST single cell clone (described in Chapter 2).
Figure 4.3: DNA-PK phosphorylation is lowered in ST expressing cells. (A) Single cell cloned U2OS cells stably expressing vector or ST were irradiated with 5 Gy and harvested at the indicated times and assessed by immunoblot with the indicated antibodies. Blot is representative of three independent experiments. Each experiment was performed with vector single cell clone 3 and a different ST single cell clone (described in Chapter 2). (B) Quantification of phospho-S2056 DNA-PKcs with Licor system. Signal intensity is normalized to total DNA-PKcs signal. Significance was determined using two-sided Student’s t-test (- p < 0.1; * p < 0.05). (C) U2OS single cell cloned cell lines that stably express vector or ST were grown on cover slips, irradiated with 5 Gy and harvested 15 minutes after IR. Coverslips were stained with primary antibody against phospho-S2056 DNA-PKcs, followed by secondary antibody conjugated to Alexa-488 and DAPI. Foci were visualized by immunofluorescence microscopy. Example image of
Figure 4.3: DNA-PK phosphorylation is lowered in ST expressing cells (Continued).

(immunofluorescence staining is shown here. (D) The number of phospho-S2056 DNA-PKcs foci images. Significance was determined using two-sided Student’s t-test (* p < 0.05). Each biological replicate was performed with vector single cell clone 3 and a different ST single cell clone (described in Chapter 2).
No interaction detected between ST and DNA-PKcs

One potential mechanism for the effect observed on the phosphorylation of DNA-PKcs is a direct interaction of MCPyV ST with DNA-PKcs or other mediators of NHEJ. Immunoprecipitation experiments testing for the interaction of ST and DNA-PKcs in the presence or absence of IR, were negative (data not shown). In addition, I reanalyzed previous MudPIT experiments performed by Dr. Jingwei Cheng and Donglim Park with less stringent parameters. These data have no indication that there is a specific interaction of ST with DNA-PKcs or other components of NHEJ. Hence, there is no evidence thus far that there is a direct interaction between ST and DNA-PKcs that would explain the difference in the observed phosphorylation of DNA-PKcs at S2056.

Activation of H2AX

To further characterize perturbations of the DNA damage response by ST, vector control or ST expressing cells were treated with increasing doses of gamma-irradiation and assessed by immunoblot for levels of γH2AX 1 hour post irradiation. ST expressing cells appeared to have slightly lower levels of γH2AX than vector control cells at all doses (Figure 4.4A). This difference was best visualized in the 2 Gy treated samples. This experiment had the same result across two replicates. It should be noted that this experiment was performed with the pooled ST expressing cells and not the single cell cloned ST cell lines. Therefore, the differences observed may be less visible due to the variable and lower levels of ST in pooled cell lines.

Next, I examined the kinetics of γH2AX induction by immunoblot. Vector control or ST expressing cells were treated with 2 Gy and assessed at 0, 1, 4, and 24 hours after irradiation.
Consistent with the dose curve in Figure 4.4A, ST expressing cell lines had lower levels of \( \gamma H2AX \) at the 1 hour time point (Figure 4.4B). However, the levels of \( \gamma H2AX \) did not persist in either the vector or the ST cell lines.

Since the immunoblot indicated that MCPyV ST could blunt the appearance of H2AX after ionizing radiation, I examined \( \gamma H2AX \) foci by immunofluorescence microscopy to determine if the portion of cells expressing \( \gamma H2AX \) was affected by the presence of ST. When scored for \( \gamma H2AX \) positive cells, I observed that the presence of ST did not change the proportion of cells that expressed \( \gamma H2AX \) over time (Figure 4.4C).
Figure 4.4: Kinetics of γH2AX after IR is slightly altered with ST. (A) Pooled U2OS cell lines stably expressing vector or ST were irradiated with 0, 2, or 4 Gy and harvested at 1 hour. Samples were analyzed by immunoblot for γH2AX and H2AX expression. Blot is representative of two independent experiments. (B) Pooled U2OS cell lines stably expressing vector or ST were irradiated with 2 Gy and harvested 1 hour after IR. Expression of γH2AX and H2AX was analyzed by immunoblot. Blot is representative of two independent experiments. (C) U2OS single cell cloned cell lines that stably express vector or ST were grown on cover slips, irradiated
Figure 4.4: Kinetics of γH2AX after IR is slightly altered with ST (Continued).

with 5 Gy and harvested at various time points after IR. Coverslips were stained with primary antibody against γH2AX, followed by secondary antibody conjugated to cy3 and DAPI. γH2AX foci were visualized using immunofluorescence microscopy. Cells were scored manually for the percentage of γH2AX positive cells. Cells with more than 5 γH2AX foci were scored as positive. Differences between vector and ST were not significant when analyzed with using two-sided Student’s t-test (p > 0.05). Each biological replicate was performed with vector single cell clone 3 and a different ST single cell clone (described in Chapter 2).
p53 and implications on cellular survival

Since we observed an overall decreased survival in ST expressing cells compared to vector cells after exposure to DNA damaging agents (Chapter 3), I decided to examine downstream effectors of DNA damage signaling. In mammalian cells, the activation of p53 and p21 are involved in growth arrest, senescence and apoptosis (Childs et al., 2014). In cells expressing vector or ST, IR led to an increase in p53 levels and phosphorylation of p53 at S15, indicating that p53 activation was occurring (Figure 4.5). However, expression of p21 was lower in ST expressing cells compared to vector, indicating that p53-mediated p21 signaling may be impaired in the presence of ST (Figure 4.5).
Figure 4.5: p21 expression is altered in cells expressing ST. U2OS single cell cloned cell lines that stably express vector or ST were irradiated with 5 Gy and harvested at the indicated times. Samples were analyzed by immunoblot for the indicated proteins. Blot is representative of three independent experiments. Each experiment was performed with vector single cell clone 3 and a different ST single cell clone (described in Chapter 2).
Discussion

MCPyV ST perturbs signaling pathways after DNA damage. In particular, we observed that cells expressing ST have lower levels of phosphorylation of DNA-PKcs at S2056 after IR. In contrast, we did not find inhibition of ATM activation after DNA damage. Therefore, ST seems to alter signaling pathways related to NHEJ but not HR.

Lower levels of phosphorylation at residue S2056, a well-characterized residue in the PQR cluster, is consistent with the inhibition of NHEJ. Previously, it had been reported that mutating residues in the PQR cluster to alanine allowed more HR than WT DNA-PKcs \cite{Cui et al., 2005, Neal et al., 2011}. In a more recent model of DNA-PK regulation, it is thought that phosphorylation in the PQR cluster is required for the initial engagement of DNA-PKcs with the site of the break \cite{Neal et al., 2014}. Therefore, the difference in phosphorylation of S2056 on DNA-PKcs in ST expressing cells is consistent with the NHEJ inhibition discussed in Chapter 3.

The mechanism for the difference in phosphorylation at S2056 is not yet clear. We could not detect the interaction of ST with DNA-PKcs in immunoprecipitation experiments. It is possible that the interaction is transient or that it is mediated by other cellular proteins that are perturbed by ST.

ATM and DNA-PK have been shown to have redundant functions in phosphorylation of proteins such as $\gamma$H2AX \cite{Stiff et al., 2004}. Therefore, the difference in total $\gamma$H2AX levels observed by immunoblot is interesting. It is possible that the alteration of DNA-PKcs regulation causes a difference in DNA-PK kinase activity that is visible on this substrate. Testing other DNA-PKcs substrates that are phosphorylated in cells could show functional differences in DNA-PK kinase activity in the presence of ST.
It is unclear if the differences in DNA-PK phosphorylation are enough to explain the extent of the NHEJ inhibition discussed in Chapter 3. However, the reporter assays in Chapter 3 are based on the formation of a single break per cell. Thus, the effect of ST may be enough to alter the balance between HR and NHEJ in these assays. It is also possible that ST affects multiple points in NHEJ and thus has a stronger phenotype than can be observed by looking only at DNA-PKcs. These perturbations to the signaling pathway could explain differences in repair by NHEJ.

Overall, in this chapter, we observed that the presence of ST altered various signaling events after DNA damage. In particular, we observed that ST inhibits the phosphorylation of DNA-PKcs at S2056, which can lead to inhibition of NHEJ. In addition, we observed that p21 levels were lower in ST expressing cells after DNA damage, although p53 was unaffected. This indicates that p21-mediated checkpoint response signaling may be altered in ST expressing cells. These two effects are consistent with the increased DNA damage sensitivity and the inhibition of NHEJ characterized in Chapter 3.
Figure 4.6: ST perturbs DNA damage response. DNA-PKcs is phosphorylated at a lower level at S2056 in ST expressing cells. The downstream phosphorylation of γH2AX, a substrate of DNA-PK, is affected. ST does not affect phosphorylation of ATM, or downstream phosphorylation of CHK2. However, ST changes the expression of p21, downstream of p53 expression.
Chapter V. Discussion
Summary of findings

In this dissertation, we sought to understand the cellular perturbations induced by Merkel Cell Polyomavirus (MCPyV) small T antigen (ST). In particular, we examined the role of MCPyV ST in the cellular DNA damage response and repair pathways.

In chapter two, we examined the potential functions of ST through predicted structure analysis and characterization of its cellular binding partners. ST was observed to interact with the PP2A phosphatase and the Tip60 complex, a cellular complex involved in histone modifications and DNA damage repair. A predicted structure of ST was generated through modeling based on the known co-crystal structure of SV40 and PP2A. With this methodology, we identified a region of ST that we called loop 4 (Figure 2.2). This approach was similar to the methodology used by the Moore and Chang groups to identify the region they call the LT stabilization domain (LSD) \cite{Kwun et al., 2013}. LSD overlaps with loop 4 in our prediction. We identified residues 86-87 in loop 4 that were necessary for binding of ST to the Tip60 complex. However, none of the point substitution mutations in ST, predicted to directly contact PP2A, were defective in binding to PP2A. Of note, we observed that epitope tags on the N-terminus of ST caused the disruption of binding to the Tip60 complex (Figure 2.1). Therefore, we were able to separate binding of ST to the Tip60 complex but could not disrupt binding to the PP2A complex without also losing binding to the Tip60 complex.

In chapter three, we examined the effect of ST on DNA damage sensitivity and repair of DSB. A smaller fraction of ST-expressing cells compared to control survived after treatment with ionizing radiation or etoposide, as measured in the colorimetric survival assay (Figure 3.1) though differences were not statistically significant. In addition, we observed that ST expressing
cells formed fewer colonies in a clonogenic survival assay than control cells in response to ionizing radiation (Figure 3.2A). In contrast, ST behaved more similarly to vector cells when treated with cisplatin and assayed in the clonogenic survival assay (Figure 3.2B). ST expressing cells also had persistent DSB after exposure to ionizing radiation, compared to vector, when measured by neutral comet assay (Figure 3.3), suggesting that ST expressing cells were defective in DSB repair. In a separate analysis that specifically measures repair by HR or by NHEJ using I-SceI based reporter repair assays, we observed that ST inhibits NHEJ but increases repair by HR (Figure 3.5). The inhibition in repair was consistent with our observation of increased persistence of DSB after damage. Interestingly, ST mutants that did not bind to the Tip60 complex behaved similarly to wild type (WT) ST in two assays, indicating that this phenotype may not be related to the relationship between ST and the Tip60 complex.

In chapter four, we examined whether ST altered DNA damage signaling after dsDNA damage. We examined the ATM and DNA-PKcs kinases that are known to be central to the DNA damage response. The activation of ATM in response to ionizing radiation in ST expressing cells appeared normal (Figure 4.2). However, DNA-PKcs phosphorylation at residue S2056 within the PQR cluster was induced to lower levels in ST expressing cells after ionizing radiation compared to control cells (Figure 4.3). Phosphorylation of residue S2056 in the PQR cluster has been reported to be required for the initial engagement of DNA-PKcs with the site of the break [Neal et al., 2014]. Also, we observed that while p53 stabilization was unaffected after ionizing radiation, we observed decreased levels of p21, indicating that the checkpoint response in ST expressing cells was altered downstream of ATM and DNA-PK signaling (Figure 4.5).
Thus, signaling pathways after exogenous DNA damage appear to be disrupted in ST expressing cells.

Taken together, these results indicate that MCPyV ST perturbs DNA damage signaling and repair. ST inhibits DNA damage repair by NHEJ and increases the sensitivity of cells to DSB. These results have implications on the viral life cycle and the initiation and treatment of MCC.

Discussion

Understanding ST functions

The study of DNA tumor viruses has led to major advances in our understanding of the molecular events that program the malignant state. Polyomavirus T antigens have revealed fundamental insights into human cancer biology. The transforming function of polyomavirus T antigens has been shown to involve targeting of cellular proteins essential for human cancer development. Furthermore, their study has led to the deciphering of signaling and growth control pathways central to viral transformation and spontaneous human cancer development. For example, the tumor suppressor p53 was initially discovered through its interaction with LT (Linzer and Levine, 1979). Study of the SV40 LT interaction with RB led to important insights into how RB regulated the cell cycle and the role of cyclin/CDKs in enabling RB to allow cell cycle progression (reviewed in DeCaprio, 2009). Indeed, SV40 LT was the first reported eukaryotic substrate of the CDK cdc2 (McVey et al., 1989). Study of Mouse polyomavirus MT led to the discovery and elucidation of the phospho-inositol 3’ kinase (PI3K) pathway and
provided key insights into the SRC tyrosine signaling cascade (reviewed in [Dilworth, 2002]). In addition to cancer, infection with polyomaviruses contribute to significant human diseases with significant morbidity and mortality including progressive multifocal leukoencephalopathy with JC polyomavirus (JCPyV), polyomavirus associated nephropathy and hemorrhagic cystitis with BK polyomavirus (BKPyV), as well as severe disfiguring skin disorders caused by human polyomavirus 7 (HPyV7) and trichodysplasia spinulosa-associated polyomavirus (TSPyV) [Arthur et al., 1986; Ho et al., 2014; Koralnik et al., 1999; Moens et al., 2011; van der Meijden et al., 2010].

The discovery of Merkel Cell polyomavirus in 2008 directly linked polyomaviruses to oncogenesis in humans [Feng et al., 2008]. The expression of ST in MCPyV positive MCC has been reported to be required for the growth but not survival of MCC cells [Shuda et al., 2011]. Thus, understanding ST functions is important in the context of human disease. From earlier studies with SV40 and MPyV, it was shown that ST can play a role in transformation of cells [Asselin et al., 1983; Asselin et al., 1984; Bikel et al., 1987; Hahn et al., 2002; Noda et al., 1986; Sleigh et al., 1978; Yu et al., 2001].

Only a few specific functions have been described for MCPyV ST. MCPyV ST expression increases 4EB-P1 hyperphosphorylation, which causes an increase in cap-dependent translation [Shuda et al., 2011]. In contrast, SV40 decreases 4EB-P1 phosphorylation, leading to the inhibition of cap-dependent translation in infection in CV-1 cells [Yu et al., 2005]. ST also acts as an inhibitor of NF-κB signaling by interacting with NF-κB essential modulator (NEMO) [Griffiths et al., 2013]. Most recently, MCPyV ST has also been shown to be involved in LT stability and LT-mediated viral replication through inhibition of FBW7 [Kwun et al., 2013].
ST may be involved in cell cycle regulation. The inhibition of FBW7 has the consequence of increasing levels of FBW7 regulated genes such as c-Myc and Cyclin E, which promotes proliferation [Kwun et al., 2013]. In addition, inhibition of ST in MCPyV-positive MCC cell lines has been reported to cause a decrease in cell cycle progression [Shuda et al., 2011]. However, a more recent report indicates that established MCC cell lines do not require ST expression for growth and that the findings by Shuda et al. may be due to off target effects of the shRNA [Angermeyer et al., 2013; 2014]. In this dissertation, we observed that transient expression of ST or expression of ST in stable cell lines did not affect cell cycle profile (Chapter 3). Thus, it is possible that MCPyV ST has effects on cell cycle in the context of the other viral proteins but not when expressed alone.

The interaction of MCPyV ST with PP2A Aβ may have functional implications for ST expression in MCC. Despite the high sequence similarity to PP2A Aα, Aβ is thought to play a functionally distinct role in the cell. Mutations in PP2A Aβ are specifically associated with some lung, colorectal, and breast cancers, as well as some melanomas [Calin et al., 2000; Tamaki et al., 2004; Wang et al., 1998]. PP2A Aβ has been implicated in the regulation of RalA; mutations that abolish this function can lead to transformation of cells [Sablina et al., 2007]. Thus, PP2A Aβ is thought to be a tumor suppressor. It is unclear if MCPyV ST inhibits PP2A Aβ in the same way that it is thought to inhibit some PP2A Aα activities. If so, it is possible that ST has an effect on cell survival and proliferation through changing the regulation of RalA. Therefore, further investigation into the functional consequences of ST binding to Aβ may potentially reveal further roles of ST in cells.
The interaction of MCPyV ST with the Tip60 complex (Chapter 2) opens up potential novel functions not shared with ST from other polyomaviruses. In this dissertation, we tested the hypothesis that the interaction of ST with the Tip60 complex is responsible for the effects of ST on the DNA damage response. The Tip60 complex is involved in the opening of structured chromatin at the site of DSB [Xu and Price, 2011]. The ST mutant 83-88A, which was unable to bind the Tip60 complex but maintained detectable binding to PP2A, behaved indistinguishably from WT ST when tested for DNA damage survival by the clonogenic survival assay (Figure 3.2A). Similarly, the ST mutants E86S/E87S ST and 86/87/92/93S ST, which have impaired binding to the Tip60 complex, behaved similarly to WT ST and were observed to inhibit the efficiency of NHEJ (Figure 3.5B). This indicates that ST binding to the Tip60 complex did not contribute to the DNA damage repair phenotype seen in ST expressing cells. The Tip60 complex has a role in chromatin remodeling that potentially makes the DSB more accessible and has been reported to contribute to both HR and NHEJ activities [Murr et al., 2006; Robert et al., 2006]. We did not specifically test for the impact of ST on chromatin accessibility in the DDR. It is possible that the effects of ST on the Tip60 complex provide conflicting signals that result in no impact on DNA repair or DDR signaling.

However, it remains possible that ST perturbs other cellular functions of the Tip60 complex. The TIP60 protein was reported to be involved in transactivation of the HIV-1 promoter and is required for HIV-1 replication [Kamine et al., 1996]. Indeed, Tip60 complex proteins were enriched in the chromatin fraction of ST expressing cells compared to vector
control cells (Figure 3.9). This enrichment in chromatin fraction may be indicative of a role for the Tip60 complex in transcriptional regulation.

**ST inhibition of NHEJ**

In this dissertation, we observed that ST perturbs the cellular DNA damage response to exogenous damage. Our data shows that MCPyV ST inhibits the repair of DSB by NHEJ but not by HR. Overall, this leads to the persistence of unresolved DSB after DNA damage and an increased sensitivity to DNA damage by IR. In addition, we observed that after γ-irradiation, ST expressing cells have lower levels of phosphorylation of DNA-PKcs at S2056, a well-characterized residue in the PQR cluster, than vector cells, indicating that ST may alter the regulation of DNA-PK.

Phosphorylation in the PQR cluster contributes to the regulation of DNA-PK activity. Mutation of residues in the PQR cluster allows more repair by HR than WT DNA-PKcs \([\text{Cui et al., 2005; Neal et al., 2011}]\). It has also been reported that phosphorylation in the PQR cluster is required for the initial engagement of DNA-PKcs with the site of the break \([\text{Neal et al., 2014}]\). In fact, the phosphorylation of DNA-PKcs at S2056 has been specifically reported to be necessary for repair by NHEJ \([\text{Chen et al., 2005}]\). Therefore, the difference in phosphorylation of S2056 on DNA-PKcs in ST expressing cells is consistent with the inhibition of NHEJ that is observed.

It is not yet clear if the presence of ST has a direct effect of the ability of DNA-PKcs to become phosphorylated at S2056. We observed no evidence for the interaction of ST with DNA-PKcs, Ku70, or Ku80 in the MudPIT analysis from our lab. In addition, we were unable to detect interaction of ST with DNA-PKcs in immunoprecipitation experiments. This does not preclude
interaction of the two proteins; it is possible that the interaction is transient or that it is mediated by other cellular proteins that are perturbed by ST.

One potential mechanism is that PP2A is involved in the regulation of DNA-PKcs and that ST disrupts this regulation. PP5 has been shown to regulate DNA-PKcs phosphorylation at T2609 and to a lesser extent at S2056 (Wechsler et al., 2004). In addition, PP5 (PPP5C) has been reported to interact with the PP2A A subunit (Lubert et al., 2001). It is possible that PP5/PP2A is indeed functional in DNA-PKcs regulation, although no reports have confirmed the interaction as biologically relevant. In okadaic acid treated cells, at PP2A-selective concentrations, DNA-PKcs activity was significantly reduced (Douglas et al., 2001). Interestingly, SV40 ST has been used as a tool to assess the role of PP2A in dsDNA break repair (Wang et al., 2009). When Wang et al. measured dsDNA breaks by pulsed-field gel electrophoresis (PFGE), they observed that SV40 ST expressing cells had more breaks (Wang et al., 2009). Interestingly, PP2A has also been implicated in the regulation of ATM by continual dephosphorylation of the activating S1981 in undamaged cells (Goodarzi et al., 2004). Since we observed normal ATM signaling in the absence of damage, ST may selectively regulate PP2A functions in the context of damage.

Polyomavirus ST-mediated alteration of cellular PP2A activity has been explored in depth with MPyV ST and SV40 ST. PP2A is a holoenzyme consisting of the scaffolding subunit A, the regulatory subunit B, and the catalytic subunit C (Janssens and Goris, 2001). SV40 ST binds directly to PP2A A whereas the interaction with the C subunit is through the interaction with the A subunit (Ruediger et al., 1992). The binding site for the B subunit on the PP2A A subunit overlaps with the binding site for ST, suggesting that ST alters PP2A activity by replacing the B subunit in the complex (Chen et al., 2007b; Cho et al., 2007; Ruediger et al.,
One example of the functional consequences of ST alteration of PP2A activity is with SV40 ST and c-MYC. SV40 ST can inhibit the PP2A-mediated dephosphorylation of c-MYC, which increases the stability of c-MYC in cells [Yeh et al., 2004]. Thus, the interaction of ST with PP2A can have dramatic effects on cellular processes.

It has been shown that MCPyV ST displaces the B56α subunit but not the B55α, B56γ3, B56δ, and B56ε subunits of PP2A, thereby affecting the specificity of the enzymatic activity [Kwun et al., 2015]. If PP2A is involved in regulating the phosphorylation of DNA-PKcs, the alteration of the regulatory subunit activity by ST could explain the lower extent of phosphorylation of DNA-PKcs S2056.

Implications for Merkel cell carcinoma

The prognosis of MCC depends on a number of factors. Patients with tumor sizes of $\leq 2$ cm and negative lymph nodes have significantly improved survival rate compared to patients with larger tumors or positive lymph nodes [Lemos et al., 2010]. In addition, detection of MCPyV virus may be an important prognostic tool. In a Finnish study of 114 tumors, researchers observed that patients with MCPyV positive tumors had a better overall survival than those with MCPyV negative tumors [Sihto et al., 2009]. A smaller study of 33 patients observed similar results, with patients with MCC containing at least one integration event of the MCPyV genome having better outcomes to treatment [Laude et al., 2010]. However, there are conflicting reports about the role of MCPyV detection in MCC survival. A newer study of 174 patients has concluded that MCPyV status was not correlated with overall MCC survival [Schrama et al.,]
If indeed the virus is correlated with better treatment response, this may be explained in part by the findings in this dissertation.

The increased DNA damage sensitivity in ST expressing cells may provide a molecular basis for the effectiveness of MCC therapies. Currently, MCC are treated depending on the stage of the disease. For stage 1 and 2 disease, therapies generally include surgery and radiation therapy (Pectasides et al., 2006). MCC is considered a highly radiosensitive tumor. In stage 3 disease, treatment is often supplemented with chemotherapy, including drugs that induce DNA damage. These drugs include doxorubicin, epirubicin, and the topoisomerase inhibitor etoposide, which can cause DNA damage (Olinski et al., 1997; Pommier et al., 2010; Tacar et al., 2013; Tai et al., 2000). Etoposide is often used in conjunction with cisplatin, which causes platinum-DNA adducts that are repaired by the Fanconi Anemia and HR pathways (Boeckman et al., 2005; Jamieson and Lippard, 1999). Thus, the increased sensitivity of ST expressing cells can contribute to the efficacy of these treatments.

The expression of ST in tumor cells may alter the sensitivity of cells to therapeutics that induce DNA damage and therefore be relevant to the prognosis of patients. Interestingly, in our assays, ST expressing cells were more sensitive than control cells to treatment with irradiation and etoposide, two methods by which MCC is treated. However, ST expression did not strongly affect sensitivity to cisplatin in our assay. Therefore, while IR and etoposide may be more effective in ST-positive cells, cisplatin may not have the same advantage in ST-positive MCC. Also, further elucidation of the mechanism by which expression of ST increases DNA sensitivity of cells may reveal more targeted treatments for MCC that may have less severe side effects than the current therapies.
Overcoming checkpoints

Rb is a key regulator of the cell cycle and is involved in the G1 to S-phase transition by regulating the function of the E2F transcription factor (reviewed in Sherr and McCormick, 2002). Loss of normal Rb function can cause unchecked cellular proliferation and p53 activation, leading to p53-dependent apoptosis (Morgenbesser et al., 1994). p53 activation leads to the transcription of multiple genes, including p21, which in turn regulates cell cycle progression after cellular stress and DNA damage (el-Deiry et al., 1993).

SV40 LT interacts with both Rb and p53 (DeCaprio et al., 1988; Reich and Levine, 1982). Similarly, in MCPyV, the interaction of LT with Rb was found to be important for LT-mediated cell cycle stimulation (Houben et al., 2012; Sihto et al., 2011). In the case of SV40 LT, the interaction of LT and p53 can inhibit the ability of p53 to induce p21 and other p53-regulated genes (Lilyestrom et al., 2006). In contrast, MCPyV LT has been reported to bind Rb but not p53 (Cheng et al., 2013). In addition, MCC usually have expression of WT p53 (Sihto et al., 2011). Thus, MCPyV must have an alternative mechanism for altering p53 function in cells for viral replication and for MCC. In chapter 4, we observed that p53 was activated and stabilized after ionizing radiation in both control and ST cell lines (Figure 4.5). However, p21 expression is lowered in ST expressing cell lines compared to control cells (Figure 4.5). Thus, it is possible that ST inhibits signaling downstream of p53 activation.

DNA damage and the MCPyV life cycle

In many cases, activation of the DNA damage response is necessary for productive viral infection. The activation of ATM has been reported to be important for the replication of the
polyomaviruses BKPyV, MPyV, and SV40 [Dahl et al., 2005; Jiang et al., 2012; Shi et al., 2005]. Similarly, MCPyV infection causes ATM to co-localize with LT, which is important for MCPyV replication [Tsang et al., 2014]. Thus, the modulation of DNA-PK mediated signaling in ST expressing cells may allow the virus to use the ATM pathway without triggering an overall DNA damage response.

Although MCC have clonally integrated MCPyV, integration into cellular DNA is not advantageous for MCPyV. In fact, MCC cell lines seem to selectively have mutations in LT, which are unable to initiate MCPyV replication at the viral origin [Kwun et al., 2009; Shuda et al., 2008]. In addition to the replication inhibiting mutations of LT, MCPyV does not have a mechanism for entering and escaping from latency.

The consequences of inhibition of NHEJ by ST for MCPyV replication is unclear. One possibility is that ST inhibition of repair by NHEJ lowers the chances of spontaneous integration into the genome. Blocking NHEJ has been shown to decrease the rate of random integration into some cells [Iiizumi et al., 2008]. In addition, the targeted integration of retrovirus by the viral integrase is thought to be mediated by the host DNA-PK pathway [Daniel et al., 1999]. Thus, inhibition of NHEJ could potentially decrease unintentional integration of viral sequences.

An alternate possibility is that ST inhibition of repair by NHEJ increases the chances of spontaneous integration into the host genome and may contribute to the potential to develop MCC. Persistent DSB may lead to random integration. For example, Duck hepatitis B virus integration has been shown at sites of dsDNA damage thorough random recombination events [Bill and Summers, 2004; Nagaya et al., 1987]. Inhibition of NHEJ may lead to repair of the break by other pathways, which may be more error prone. One pathway that may be upregulated
in the absence of NHEJ is alternative NHEJ (a-NHEJ), which has been shown to favor translocations in cells (Reviewed in Betermier et al., 2014). The fidelity of NHEJ also seems to be highly dependent on KU80 function [Guirouilh-Barbat et al., 2004].
Figure 5.1: Model for ST mediated perturbations of DNA damage response. ST causes the inhibition of DNA damage repair through altering the phosphorylation of PP2A, which leads to an inhibition of NHEJ and decreased DSB repair. In addition, the effect of ST on p53-p21 mediated checkpoint response can contribute to the overall DNA damage sensitivity phenotype.


Bouquet, F., Muller, C., and Salles, B. (2006). The loss of gammaH2AX signal is a marker of DNA double strand breaks repair only at low levels of DNA damage. Cell Cycle 5, 1116-1122.


PKcs phosphorylations at the Thr-2609 cluster upon DNA double strand break. The Journal of biological chemistry 282, 6582-6587.


Hart, L.S., Yannone, S.M., Naczki, C., Orlando, J.S., Waters, S.B., Akman, S.A., Chen, D.J.,
strand break repair and radiosensitizes human tumor cells in an E1B-55K-independent manner.
The Journal of biological chemistry 280, 1474-1481.

Hirao, A., Kong, Y.Y., Matsuoka, S., Wakeham, A., Ruland, J., Yoshida, H., Liu, D., Elledge,

Ho, J., Jedrych, J.J., Feng, H., Natalie, A.A., Grandinetti, L., Mirvish, E., Crespo, M.M., Yadav,
D., Fasanella, K.E., Proksell, S., et al. (2014). Human Polyomavirus 7-Associated Pruritic Rash

Houben, R., Adam, C., Baeurle, A., Hesbacher, S., Grimm, J., Angermeyer, S., Henzel, K.,
in Merkel cell polyomavirus large T antigen is required for promoting growth of Merkel cell

Houben, R., Shuda, M., Weinkam, R., Schrama, D., Feng, H., Chang, Y., Moore, P.S., and

transduces the DNA-damage signal via histone ubiquitylation and checkpoint protein assembly.
Cell 131, 901-914.

(2008). Impact of non-homologous end-joining deficiency on random and targeted DNA

Ikura, T., Tashiro, S., Kakino, A., Shima, H., Jacob, N., Amunugama, R., Yoder, K., Izumi, S.,
Kuraoka, I., Tanaka, K., et al. (2007). DNA damage-dependent acetylation and ubiquitination of
H2AX enhances chromatin dynamics. Molecular and cellular biology 27, 7028-7040.

DNA Adducts. Chemical reviews 99, 2467-2498.

Janssens, V., and Goris, J. (2001). Protein phosphatase 2A: a highly regulated family of
serine/threonine phosphatases implicated in cell growth and signalling. The Biochemical journal
353, 417-439.


damage checkpoint signal transduction elicited by herpes simplex virus infection. The Journal of biological chemistry 280, 30336-30341.


