

# Large T and small T antigens of Merkel cell polyomavirus

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Merkel cell polyomavirus (MCV) is the etiological agent of Merkel cell carcinoma (MCC), a rare and highly lethal human skin cancer. A natural component of skin flora, MCV becomes tumorigenic only after integration into the host DNA together with specific mutations to the viral genome. Research on MCV large T (LT) and small T (sT) antigens, the only viral products expressed in MCC, shows that these major oncoproteins not only possess biochemical functions found in common with other polyomavirus T antigens, but also demonstrate new cellular targets not described in previous polyomavirus models. This review provides a map of the relevant functional motifs and domains in MCV T antigens that have been identified, highlighting their roles in tumorigenesis.

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## Introduction

Merkel cell polyomavirus (MCV) is the newest member of the short list of human cancer viruses [1,2], and is the only known human polyomavirus confirmed to be oncogenic [3–17]. Although MCV was only discovered in 2008, polyomavirus research dates back over a half-century, beginning with the isolation of murine polyomavirus (MuPyV) [18] and later simian vacuolating virus 40 (SV40) [19,20]. These polyomaviruses have provided invaluable insights into our mechanistic understanding of tumor and cell biology. Polyomaviruses have small genomes (~5 kb) comprised of early and late coding regions, separated by a noncoding regulatory region (NCRR). The early region contains the T (“Tumor”) antigen gene locus [21], from which multiple, alternatively-spliced RNA transcripts are generated. MCV expresses four unique gene products from this early coding region:

the large T (LT), small (sT), and 57 kT antigens along with a product from an alternate frame of the LT open reading frame (ALTO) [22] (Figure 1). In natural polyomavirus lytic infection, a sequential expression of early antigens followed by late capsid proteins is seen. By contrast, MCV-associated tumorigenesis is characterized and mediated by the sole expression of LT and sT antigens [21,23]. This review will present a biochemical map of the functionally relevant motifs and domains within LT and sT, the two major oncoproteins of MCV.

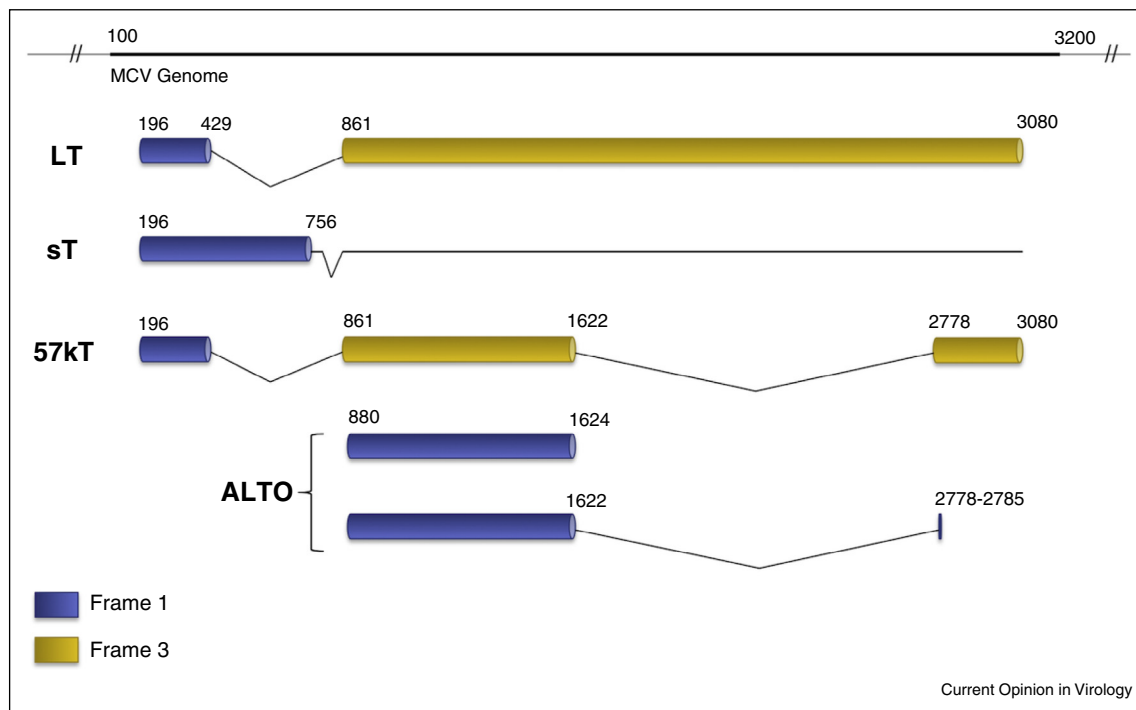
## Large T antigen

The LT antigens of polyomaviruses contain a number of common motifs and domains important for facilitating the viral life cycle [24]. In the context of oncogenesis, some of these elements also have the effect of disabling tumor suppressor pathways, for example by targeting Rb and p53 [21]. The LT antigen of MCV encodes many of these conserved features as well as a few unique ones (Figure 2).

The N-terminal end of MCV LT (1–70 aa) contains the DnaJ domain [24] comprised of the CR1 (13–17 aa) motif followed by the HPDKGG hexapeptide sequence responsible for Hsc70 binding (42–47 aa) [24,25]. Kwun *et al.* confirmed that MCV LT interacts with Hsc70, and by disrupting this interaction with a point mutation, they showed the necessity of the DnaJ domain for MCV replication *in vitro* [26].

Between the first exon and the OBD (~100–300 aa) lies a stretch of sequences that contains a conserved LXCXE motif and nuclear localization signal [27], but otherwise bears little homology to other polyomaviruses. This region, designated the MCV T antigen unique region (MUR) contains a binding motif for the vacuolar sorting protein Vam6p. The LT–Vam6p interaction, which can be ablated by mutation of a single tryptophan residue at position 209, results in the nuclear sequestration of this cytosolic protein and disrupts lysosomal clustering [28]. Although Vam6p interaction appears to be unique to MCV, the site of this interaction parallels the site for Bub1 interaction in SV40 LT, which also depends on the presence of tryptophan residues and modulates SV40 LT-mediated transformation by overriding the mitotic spindle checkpoint [21,29]. In an *in vitro* replication assay using an infectious molecular clone of MCV mutated at position 209, Feng *et al.* demonstrated that loss of LT–Vam6p binding leads to enhanced viral replication compared to a wild-type control [30]. It is possible that in the

Figure 1



Gene products of the early coding region. Multiple gene products are expressed from the early coding region of the MCV genome as a result of alternative splicing of RNA transcripts. Splicing patterns for each gene product are depicted. LT, sT, and 57 kT represent the main T antigens expressed by MCV, which share a common first exon sequence. An alternate frame of the LT open reading frame (ALTO) is +1-shifted with respect to the second exon of LT. A single microRNA named MCV-miR-M1 is also encoded by MCV whose mature sequence is antisense to 1215–1236 nt in the viral genome.

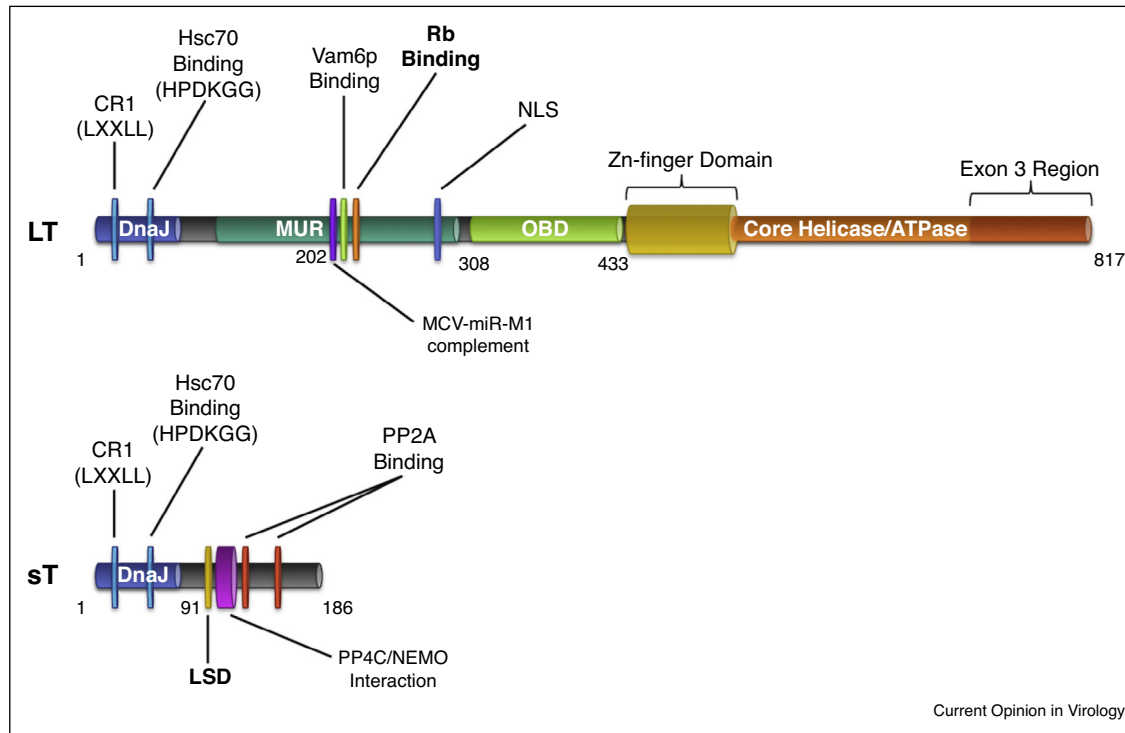
natural life cycle of MCV, LT–Vam6p interaction inhibits or minimizes viral reactivation, a potential form of ‘viral latency’. SV40 miRNA has been proposed to serve a similar autoregulatory function by inhibiting SV40 LT expression [31]. MCV encodes a miRNA that may have a similar function and may augment Vam6p-related replication silencing [32,33]. Whether or not Vam6p targeting is also important in tumorigenesis is not presently known.

The Rb-binding (LXCXE) motif of LT (212–216 aa) embedded in the MUR lies directly adjacent to the Vamp6-binding site (209 aa). This highly conserved sequence across polyomaviruses allows for dysregulation of E2F-mediated transcription and drives cells into S-phase [21,34<sup>\*</sup>]. Rb-binding and subsequent upregulation of E2F target genes such as cyclin E has been confirmed for MCV LT [27,35<sup>\*\*</sup>]. Houben *et al.* showed the importance of the LXCXE motif in the proliferation of MCC tumor cells, since complementation with an Rb-binding mutant of LT after RNAi-mediated T antigen depletion in MCC cell lines was incapable of rescuing cell growth [36<sup>\*\*</sup>]. A novel biochemical function was mapped to this region when Arora *et al.* observed that upregulation of the anti-apoptotic protein survivin upon LT expression is dependent on

the presence of and intact LXCXE motif [35<sup>\*\*</sup>]. Targeting LT-mediated survivin upregulation represents a viable therapeutic option, supported by *in vivo* studies where pharmacologic inhibition of survivin lengthened the survival of mice bearing MCC xenografts [35<sup>\*\*</sup>,37<sup>\*</sup>].

The C-terminal portion of MCV LT contains several crucial elements required for viral replication. The OBD of LT (308–433 aa) is responsible for recognition and binding of a minimum 71-bp origin of replication in the MCV NCRR [28,38]. Diaz *et al.* observed that phosphorylation of LT upstream of the OBD at residues T297 and T299 decreases the origin-binding affinity of LT and negatively affects replication initiation [39]. Following the OBD is a zinc-finger motif (437–528 aa) that is contained in the core polyomavirus helicase/ATPase domain (441–817 aa), both of which are important for LT oligomerization and initiation of replication [34<sup>\*</sup>]. In addition to replicase activity, the helicase domain in SV40 contains a bi-partite sequence that can directly bind p53 and prevent transcription of its target genes, allowing for the evasion of senescence or apoptosis [21,24,34<sup>\*</sup>,40–42]. Currently, there is no evidence that either full length or truncated forms of MCV LT directly bind p53 [43,44<sup>\*</sup>].

Figure 2



Functional domains of MCV LT and sT antigens. Schematic representation of LT and sT antigens of MCV highlights the location of several motifs and domains that are important in the viral life cycle and tumorigenesis. Regions with a key role in tumorigenesis are bolded. LT contains a DnaJ domain at its N-terminus, followed by the MUR, which contains the binding motifs for Vam6p and Rb family members as well as an NLS. The site of complementarity for the single microRNA encoded by the MCV genome, MCV-miR-M1 is also found in the MUR. The C-terminal portion of LT contains the OBD and the core helicase/ATPase domain, which contains the zinc-finger domain and the region corresponding to Exon 3 of 57 kT. sT shares exon 1 with LT, followed by the LT-stabilization domain (LSD) and sites for PP4C and PP2A interaction.

One of the most striking features of the LT antigen of MCV is that the C-terminal domains are consistently truncated by tumor associated polymorphisms, but and the N terminus up to the Rb-binding motif are always intact [25,45]. The short NLS of MCV LT that lies between these two regions (277–280 aa) was initially believed to also be conserved in tumors [27], but recent evidence indicates that the NLS is not preferentially preserved in MCC [43,46]. In the cases where the NLS is eliminated by tumor-specific mutations to LT (as in cell lines MCC350, MCCL-11, and MCCL-12) nuclear localization is not lost, but rather both nuclear and cytoplasmic distribution are observed [43].

This signature pattern of C-terminal truncation indicates a selective pressure for the elimination of viral replicative elements in tumor development since a full-length LT could initiate unlicensed replication at the site of integration in the host genome leading to a DNA damage response (DDR) and cell death [27]. This is not unlike other polyomaviruses (or other tumor viruses) in which replication competency is lost or suppressed in the setting of tumorigenesis [47–49]. This notion is supported by Li

*et al.* who observed a DDR induced by MCV infection which was mapped to the core helicase region of LT. The expression of a LT with intact and functional helicase induces ATR and subsequent p53 activation in multiple cell lines [50\*] leading to cell cycle arrest and decreased proliferation. There exists additional pressure for C-terminal truncation of LT that has been mapped to the very C-terminal end of the protein, corresponding to the third exon of the protein, corresponding to the third exon of 57 kT, which is shared by LT (717–817 aa). Upon retroviral transduction of an exon 3 construct, Cheng *et al.* noticed retardation of cell growth in an established MCC cell line as well as in human fibroblasts immortalized by SV40 [44\*]. Phosphorylation of LT at S816 by ATM likely contributes to this anti-proliferative effect [51]. Altogether, this evidence suggests that the C-terminal portion of LT generally has an anti-proliferative effect, necessitating its deletion in tumorigenesis.

### Small T antigen

Although MCV LT and sT share exon 1 of the T antigen locus, and the DnaJ domain of LT is required for replication, the functional significance of this domain in sT is unknown. It has been shown that mutating Hsc70 binding

on sT does not interfere with its effect on MCV viral replication [26] or the *in vitro* transformative activity of sT [52]. This is similar to SV40 in which the DnaJ domain of LT sT is known to bind Hsc70 [53], but this function also does not impact tumorigenesis [21,54].

The primary functions of sT antigens in the prototypic polyomaviruses SV40 and MuPyV has been long attributed to modifying Akt-mTOR signaling via their interactions with protein phosphatase 2A (PP2A) [55–57], a heterotrimeric complex comprised of ABC subunits. sT antigens bind to the A and C subunits of PP2A to displace a variety of modular B subunits [21,57], but the consequences of these interactions and their requirement in tumorigenesis varies between different polyomavirus species [58]. Both SV40 and MuPyV sT antigens have been shown to have broad effects on cell cycle progression, survival, and differentiation that are dependent on PP2A [59,60]. The primacy of PP2A targeting in other polyomavirus sT antigens contrasts directly with MCV sT, whose PP2A interacting domains (119–124 and 147–152 aa) are dispensable for its *in vitro* and *in vivo* transformative activity [52,61\*\*]. Due to limited B subunit targeting (B56 $\alpha$  only), MCV sT instead relies on its other functionalities separate from PP2A binding as a key oncoprotein for MCC [62]. MCV sT expression alone, independent of LT, is responsible for *in vitro* transformation of rodent fibroblasts in soft agar and focus formation assays [52], and sT is required for continued cell proliferation in MCC [63]. Recent work by Verhaegen *et al.* has shown that MCV sT has *in vivo* proliferative activity when expressed in mice [61\*\*].

A major feature of MCV sT is a domain spanning 74–98 aa in intron 1 that is predicted to encode for an exposed, unstructured loop remotely located from PP2A binding sites [64\*\*]. Scanning alanine mutagenesis in this loop has identified a region from 91 to 95 aa termed the LT-Stabilization Domain (LSD). This domain is responsible for inhibiting the SCF<sup>Fbw7</sup> E3 ubiquitin ligase [64\*\*]. Since LT is a target of Fbw7, co-expression of sT prevents LT degradation, increases steady-state LT levels and has been shown to enhance viral replication [64\*\*]. sT targeting of Fbw7 not only regulates LT levels during natural infections, sT also stabilizes truncated tumor T antigens and other SCF<sup>Fbw7</sup> substrates, including c-Myc and cyclin E, contributing to carcinogenesis. The importance of the LSD is evidenced by the fact that when mutated, sT loses its *in vitro* transformation activity [64\*\*].

In addition to Fbw7, an intact sT LSD domain is required for targeting 4E-BP1, the major regulator of eukaryotic cap-dependent translation. In its active, hypophosphorylated form, 4E-BP1 directly binds and inhibits eIF4E from recruiting 40S ribosomal assembly at the 5' cap of mRNA and inhibits translation. Mammalian target of

rapamycin complex 1 (mTOR1)-mediated phosphorylation of 4E-BP1 releases 4E-BP1 from eIF4E allowing translation initiation to proceed. sT expression has been shown to result in increased hyperphosphorylated 4E-BP1 that independent of mTORC1 signaling [52]. The precise mechanism for sT-mediated 4E-BP1 hyperphosphorylation is not defined at present, but is independent of PP2A and Fbw7 interaction [64\*\*].

Griffiths *et al.* through a mass spectrometric approach have revealed that MCV sT also interacts with protein phosphatase 4C (PP4C) at a region (95–111 aa) directly adjacent to the LSD at 91–95 aa [65\*]. MCV sT binding of PP4C or PP2A A $\beta$  targets the NF- $\kappa$ B essential modulator (NEMO) protein and prevents nuclear translocation of NF- $\kappa$ B, inhibiting NF- $\kappa$ B-mediated transcription [65\*]. PP4C binding is also important for the induction of a highly motile cell phenotype that may correspond to the highly metastatic nature of MCC [66].

## Conclusions

In six short years, the map of MCV has transitioned from a simple sketch to a more intricate schematic with detailed characterizations of the viral gene products and how they impact tumorigenesis. Unearthing the functional aspects of the T antigens has aided in identifying novel targets for MCC treatment, spurring the development of MCV-directed vaccines and therapeutics [67–70]. Still, there exist uncharted activities associated with MCV infection that warrant additional characterization for a more comprehensive understanding of this virus and its role in MCC. As previous research on polyomaviruses has contributed fundamental concepts in cell and cancer biology, continued efforts in elucidating the mechanisms of MCV pathogenesis may uncover similar discoveries that extend beyond the context of MCC.

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