



Molecular subtyping and vulnerability exploitation in Merkel cell carcinoma

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Molecular subtyping and vulnerability exploitation in Merkel cell carcinoma

A dissertation presented

by

Thomas C. Frost

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Virology

Harvard University

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*Molecular subtyping and vulnerability exploitation in Merkel cell carcinoma***Abstract**

Merkel cell carcinoma (MCC) is an aggressive neuroendocrine (NE) carcinoma of the skin with limited therapeutic options. Currently, MCC is divided into two subtypes based upon the presence or absence of clonally integrated Merkel cell polyomavirus (MCPyV) within the host-cell genome. These subtypes are genetically and etiologically distinct: virus-negative MCC (MCCN) are heavily mutated including specific inactivation of the RB transcriptional corepressor 1 (*RB1*) and tumor protein p53 (*TP53*) tumor suppressors while virus-positive MCC (MCCP) contain few mutations but express viral oncoproteins that functionally inhibit RB1 and p53. Despite these independent etiologies, MCCP and MCCN are considered clinically indistinguishable due to overlapping morphological and immunohistological characteristics paired with similar prognoses and responses to therapy. As such, increased molecular characterization of MCC could lead to the identification of therapeutically-relevant subtypes which may be exploitable for more targeted and efficacious treatment.

In Chapter 2, we identify inosine monophosphate dehydrogenase (IMPDH) proteins as critical and druggable targets in *TP53*-wildtype MCC. We found that inhibition of IMPDH led to the rapid depletion of DNA, but not RNA, synthesis in addition to the accumulation of replication stress (RS) and p53-dependent apoptosis. Building on these results, we combined IMPDH and ataxia telangiectasia and Rad3-related (ATR) inhibitors to uncouple RS from the ATR-dependent replication checkpoint and induce genome-wide double-stranded DNA (dsDNA) breaks and cellular death independent of *TP53*-status. In this context, we identified *TP53*-dependent and -independent therapeutic vulnerabilities exploitable in MCC with currently available therapeutics.

In Chapter 3, we used bulk and single-cell sequencing modalities to characterize the transcriptional landscape of patient-derived tumor biopsies. In these samples, we identified heterogeneity in NE gene expression that was negatively correlated with Hippo pathway transcription factors Yes1-associated transcriptional regulator 1 (YAP1) and WW-domain containing transcription regulator 1

(WWTR1). We observed this reciprocal expression at the protein and RNA levels, in patient-derived tissues, patient-derived and established cell lines, in bulk samples as well as in single-cells. This led us to categorize MCC into either NE-hi or NE-lo subtypes based on the expression of NE genes or YAP1 and WWTR1, respectively. Mechanistically, we found that induction of YAP1 and WWTR1 in NE-hi MCCP cells suppressed expression of MCPyV oncoproteins to reinstate RB1-dependent cell-cycle arrest. As a result, we identified additional NE-based subtypes of MCC which could be exploited with current NE- or non-NE-targeted therapeutics in addition to uncovering a novel YAP1- and WWTR1-dependent MCPyV oncoprotein restriction mechanism.

Taken together, this work has increased the repertoire of molecular subtypes in MCC to include *TP53*- and NE-status in addition to identifying and validating clinically targetable subtype-specific vulnerabilities. These findings support the growing push towards personalized medicine in MCC and suggest the importance of accounting for *TP53*- and NE-status throughout clinical course. Unexpectedly, this work has also unlocked a potential model for the cell-of-origin for MCCP oncogenesis and MCPyV infection, a better understanding of which could lead to the development of clinically-relevant disease models.

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Chapter 1: Introduction

Merkel cell carcinoma (MCC) is a neuroendocrine (NE) carcinoma of the skin thusly named due to histological similarities with normal mechanosensory Merkel cells (Sibley et al., 1980; Toker, 1972). First described in 1972, MCC is a highly aggressive cancer whose incidence has rapidly increased in recent years and for which effective treatment remains elusive (Lemos et al., 2010; Paulson et al., 2018; Toker, 1972; Villani et al., 2019). In 2008, the discovery of clonally integrated Merkel cell polyomavirus (MCPyV) DNA in a subset of tumors redefined the classification of MCC into virus-positive (MCCP) and -negative (MCCN) subtypes (Feng et al., 2008). These MCCP and MCCN subgroups are genetically and etiologically distinct: MCCP express viral oncoproteins to drive oncogenesis, while MCCN are transformed through the rampant accumulation of UV-induced mutations (Becker et al., 2017). Despite these clear molecular differences, MCCP and MCCN cause indistinguishable cancers under current clinical assessment (Becker *et al.*, 2017). As a result, treatment options for MCC are not delineated based upon viral subtype (Harms et al., 2018). Furthermore, no molecular markers that predict prognosis or response to therapy are established (Becker *et al.*, 2017). As such, the identification of molecular characteristics which better define MCC subtypes could enhance stratification of sensitive patient populations for targeted therapy. In this section, we summarize the molecular and etiological features which are currently used to define MCC subtypes. We also outline current treatment strategies and shortcomings. Finally, we introduce two main avenues for identification of novel MCC subtypes with implications for therapeutic interventions.

Distinct molecular and etiological characteristics of MCCP and MCCN subtypes

MCCP is defined by the clonal integration of MCPyV genetic material into the host-cell genome of MCC (Figure 1.1) (Feng *et al.*, 2008). Similar to other human polyomaviruses (HPyV), MCPyV is a small non-enveloped double-stranded DNA (dsDNA) virus that is part of the normal human microflora and typically causes benign, though life-long infection (DeCaprio, 2017). Despite common infection, the cell type or types which can host MCPyV infection have not been firmly established (Becker *et al.*, 2017). In any case, MCPyV is the only known HPyV comprehensively demonstrated to cause human cancer (Dalianis and Hirsch, 2013; Poulin and DeCaprio, 2006). It has been speculated that this activity depends

on integration of viral DNA into the host-cell genome (Dalianis and Hirsch, 2013) as is observed for the related small non-enveloped dsDNA virus, human papilloma virus (HPV), in cervical cancer (Wentzensen et al., 2004). Integration of additional HPyVs, including the JC and BK polyomaviruses in brain and prostate cancer, respectively, have recently been observed (Del Valle and Pina-Oviedo, 2019; Kenan et al., 2015; Wang et al., 2020). However, these results are sparse and require mechanistic validation of viral-mediated oncogenesis.

Currently, the mechanism by which MCPyV genetic material becomes embedded into the host-cell genome is unknown. Indeed, no integrase is encoded by the virus and integration is non-essential for the polyomavirus lifecycle (DeCaprio, 2017). It has been hypothesized that fragmentation of the MCPyV genome during normal replication could present sections of viral DNA for microhomology-mediated end joining (MMEJ) and subsequent integration (Harms *et al.*, 2018; Starrett et al., 2020). In agreement with this idea, significant enrichment of 4-, 5-, and 7-mer sequence microhomology between the human and MCPyV genomes was observed at the integrated fusion junctions (Starrett *et al.*, 2020). Regardless of mechanism, MCPyV integration is often concurrent with truncation of specific viral coding regions (Shuda et al., 2008; Starrett *et al.*, 2020).

At a minimum, the integrated MCPyV genome retains the non-coding control region (NCCR), a truncated form of the large tumor antigen (LT) and the full-length small tumor antigens (ST; Figure 1.1) (DeCaprio, 2017). The NCCR contains the origin of replication, an enhancer element, and distinct early and late promoters (Kwun et al., 2009). The early promoter element drives expression of LT and ST (collectively TAGs) which are essential for the survival and proliferation of MCCC (Houben et al., 2010;

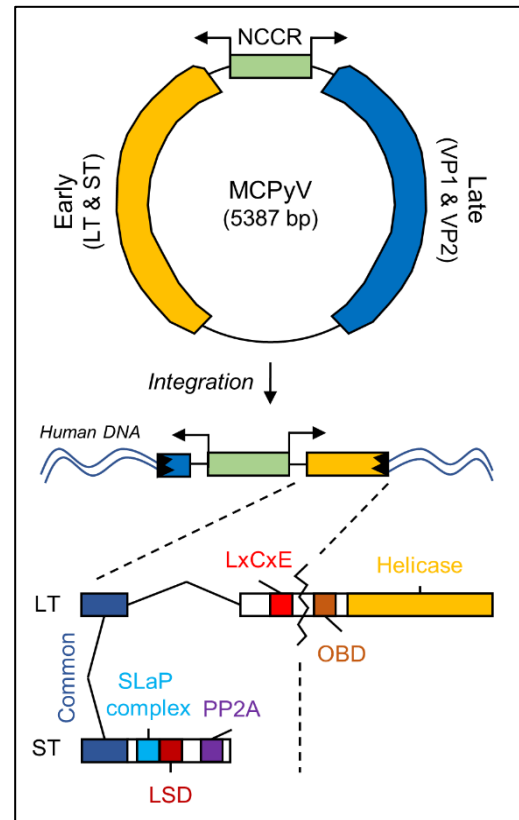


Figure 1.1 MCPyV genome organization in MCCC. (A) Normal structure and transcriptional units of the genomic MCPyV episome. (B) Organization of integrated MCPyV and human genomes. Note typical genetic truncation of early and late transcriptional units (zig-zag). Only expression of truncated LT containing the LxCxE motif and the full-length ST are conserved in all known MCCC tumors and cell lines.

Shuda et al., 2011). LT and ST open reading frames (ORFs) exist as a single transcriptional unit that is alternatively spliced into individual transcripts (DeCaprio and Garcea, 2013). The late promoter drives expression of the viral capsid proteins (viral protein 1/2 (VP1/2)), whose coding regions are typically lost upon integration and are not usually expressed in MCCC (Starrett *et al.*, 2020).

During the typical MCPyV lifecycle, LT functions as an essential replication factor by binding the viral origin of replication and functioning as a helicase to facilitate genome replication (DeCaprio and Garcea, 2013). In addition, LT contains an LxCxE motif essential for binding and inhibiting the activity of cell-cycle regulating “pocket” protein RB transcriptional corepressor 1 (RB1) (DeCaprio and Garcea, 2013; Hesbacher et al., 2016). Normally, RB1 controls cell-cycle progression by binding and inhibiting the “activating” subset of E2F transcription factors (E2F1/2/3) whose target genes are essential to drive the transition from G1 into S cell-cycle phases (Cobrinik, 2005). In this manner, RB1 functions as a tumor suppressor to prevent unscheduled entry into the cell-cycle. As a result, functional or genetic inactivation of RB1 activity is a critical requirement for oncogenesis in a variety of cancers (Dyson, 2016). In MCCC, expression of LT can overcome the G1/S cell-cycle checkpoint and promote aberrant proliferation through physical inhibition of RB1 via the LxCxE motif (Houben et al., 2012; Shuda *et al.*, 2008). Indeed, proliferative loss upon knockdown of LT can be rescued with simultaneous knockdown of RB1 in MCCC cell lines (Hesbacher *et al.*, 2016). Upon integration in MCCC, the origin binding and helicase domains of LT are typically lost, thereby rendering the integrated MCPyV genome replication defective (Shuda *et al.*, 2008; Starrett *et al.*, 2020). However, at a minimum, a functional LxCxE motif is present within LT proteins expressed in all observed MCCC tumors and cell lines (Starrett *et al.*, 2020), presumably to inhibit the tumor suppressive functions of RB1.

ST has numerous interaction partners of which several critical factors will be summarized here. ST interacts with the A and C subunits of protein phosphatase 2 A (PP2A) to displace the substrate-targeting B subunit. In this way, ST is thought to misdirect or inhibit PP2A substrate specificity, although no specific targets have been identified (DeCaprio, 2017; Pallas et al., 1990; Shuda *et al.*, 2011). ST also retains a LT-stabilizing domain (LSD) found to bind the E3 ubiquitin ligase Skp1, Cul1 and F-Box (SCF) complex containing the F-box and WD repeat domain-containing 7 (FBW7) targeting subunit (Kwun et al., 2013; Welcker and Clurman, 2008). In this context, ST was found to bind and inhibit the interaction of

FBW7 and LT, thereby preventing poly-ubiquitination and proteasomal degradation of LT (Kwun *et al.*, 2013). Recently our laboratory has uncovered an additional domain of ST that can form an interaction with MYCL proto-oncogene (MYCL) and the entire E1A binding protein p400 (EP400) histone remodeling complex to transactivate specific gene expression (Cheng *et al.*, 2017). One critical target of this ST-MYCL-EP400 (SLaP) complex is the MDM2 proto-oncogene (MDM2), an E3 ubiquitin ligase essential for controlling p53 (*TP53*) protein levels in the cell (Cheng *et al.*, 2017; Park *et al.*, 2019). As “guardian of the genome” p53 functions as a transcription factor to integrate and respond to pleiotropic sources of cellular stress, especially in the context of DNA damage (Hafner *et al.*, 2019; Kasthuber and Lowe, 2017). In response to DNA damage, the serine 15 (Ser15) residue of p53 is phosphorylated by several kinases involved in the DNA damage response (DDR) (Banin *et al.*, 1998; Shieh *et al.*, 1997; Tibbetts *et al.*, 1999). This phosphorylation prevents the association between MDM2 and p53, thereby promoting p53 accumulation in the stressed cell. Subsequently, high levels of p53 can induce cell-cycle arrest and promote apoptosis through upregulation of cyclin dependent kinase inhibitor 1A (*CDKN1A* or p21) or BCL2 binding component 3 (*BBC3* or PUMA), respectively. As a result of these severe anti-proliferative effects, *TP53* is the most commonly mutated gene in cancer (Kandoth *et al.*, 2013). By initiating formation of the SLaP complex, ST was shown to suppress p53 protein levels through transactivation of MDM2 (Park *et al.*, 2019). Supporting this idea, amplification of MDM2 is a common mechanism by which cancers control p53 levels (Hou *et al.*, 2019).

At a minimum, all known MCCC tumors and cell lines express the MCPyV TAGs to functionally inhibit the key tumor suppressors RB1 and p53 to support tumorigenesis. As a result, *RB1* and *TP53* are near-universally wildtype in MCCC (Knepper *et al.*, 2019; Starrett *et al.*, 2020). Importantly, however, functional TAG-mediated inhibition of p53 and RB1 can be overcome for therapeutic benefit. For example, chemical inhibition of MDM2 has been demonstrated to upregulate p53 levels and enforce cytotoxicity in *TP53*-wildtype MCCC *in vitro* and *in vivo* (Ananthapadmanabhan *et al.*, 2022; Park *et al.*, 2019). Indeed, the initiation of a Phase 1b/2 clinical trial of an MDM2 inhibitor (KRT-232) for treatment of *TP53*-wildtype MCC (NCT03787602) suggests the clinical potential of this approach. Taken together, the decoupling of TAG-mediated inhibition of RB1 and p53 is an attractive but emerging therapeutic strategy in MCCC.

As opposed to MCPyV-mediated oncogenesis in MCCP, MCCN is thought to be caused by the collective accumulation of mutations through excessive sun exposure. Indeed, MCCN contains a 25 to 90-fold increase in somatic mutations as compared to MCCP concurrent with a UV-induced mutational signature (Becker *et al.*, 2017; Knepper *et al.*, 2019; Starrett *et al.*, 2020). In addition to single nucleotide variations, MCCN has increased inter- and intrachromosomal translocations as well as increased copy number variations as compared to MCCP (Starrett *et al.*, 2017). This broadly increased mutational load in MCCN typically includes inactivating mutations in *RB1* and *TP53* (Knepper *et al.*, 2019; Starrett *et al.*, 2020) which cannot be overcome through functional upregulation of RB1 or p53, respectively. Furthermore, due to the lack of cell-of-origin for MCC, the specific mutational events necessary for the formation of MCCN are unclear. Taken together, MCC is genetically and etiologically stratified into MCCP and MCCN subtypes based upon the negative correlation of integrated MCPyV and a high UV-induced mutational burden.

Subtype deconvolution and immunohistopathological markers of MCC

Although distinct molecular differences between MCCP and MCCN subtypes exist, the presence of MCPyV is not typically assessed in the clinic (Becker *et al.*, 2017). MCPyV can be detected through several basic modalities including PCR-based amplification, whole-genome sequencing, hybrid-capture and targeted sequencing of viral DNA or RNA-seq and immunohistochemistry (IHC) for expression of TAg (Moshiri *et al.*, 2017; Slevin *et al.*, 2020; Starrett *et al.*, 2017; Starrett *et al.*, 2020). However, results from these assays are not always concordant (Moshiri *et al.*, 2017; Sihto *et al.*, 2009). Furthermore, there are numerous conflicting reports on whether MCPyV-status has prognostic relevance on disease outcomes, especially when additional variables such as age, sex, immunosuppression, and disease staging are included (Handscheil *et al.*, 2010; Moshiri *et al.*, 2017; Nardi *et al.*, 2012; Schrama *et al.*, 2011; Sihto *et al.*, 2009). Identification of subtype-specific treatment sensitivities are confounded by the lack of MCPyV testing in larger retrospective studies (Iyer *et al.*, 2016; Tai *et al.*, 2000; Voog *et al.*, 1999), likely due to the datasets being compiled before the discovery of MCPyV in 2008 (Feng *et al.*, 2008). More recent work which identified patient MCPyV-status found no observable differences in prognosis or responses to therapy between the subtypes (Kaufman *et al.*, 2016; Nghiem *et al.*, 2016), although

updated first-line treatment regimens and low samples sizes also confounded these datasets. As a result, it is currently unclear if prognostic or treatment-related differences exist between the MSCP and MCCN subtypes.

Despite disparate genetics and etiology, all other currently assessed molecular markers are considered to be conserved between the MCPyV-defined MCC subtypes (Becker *et al.*, 2017). MCC is a small-blue-round-cell-type tumor containing small, uniformly circular cells with minimal cytoplasm and “salt-and-pepper” chromatin (Pulitzer, 2017). As observed in other NE cancers, MCC stains positively for common NE markers such as chromogranin (CHGA) or synaptophysin (SYP), and various sizes of neurofilaments (NEFL/M/H) (Becker *et al.*, 2017). More specifically, MCC uniformly expresses SRY-box transcription factor 2 (SOX2) and atonal BHLH transcription factor 1 (ATOH1) (Gambichler *et al.*, 2017; Laga *et al.*, 2010), transcription factors critical for normal Merkel cell development (Perdigoto *et al.*, 2014). SOX2 and ATOH1 transcriptional activity is also essential for survival of MSCP (Cheng *et al.*, 2017). In addition, MCC broadly expresses cytokeratins including those expressed in normal Merkel cells such as cytokeratin 8 (KRT8) and cytokeratin 20 (KRT20) (Becker *et al.*, 2017). The “gold standard” for differential diagnosis of MCC is KRT20, which is positive in ~90% of all MCC tumors (Bobos *et al.*, 2006; Chan *et al.*, 1997; Scott and Helm, 1999) and whose expression in the skin is limited to normal Merkel cells (Chan *et al.*, 1997; Moll *et al.*, 1995). The ~10% of MCC without broad expression of KRT20 require additional immunophenotyping and correlation with clinical markers of MCC for effective diagnosis (Becker *et al.*, 2017). The consequences or mechanism for the lack of KRT20 expression within MCC tumors is currently unclear.

Therapeutic options for treatment of metastatic MCC are limited

MCC is an especially aggressive cancer with a high rate of mortality and recurrence relative to other skin malignancies. Assessment of retrospective analyses showed high disease-specific mortality rates (33-46%) for MCC (Schadendorf *et al.*, 2017). As compared to melanoma, a more common form of skin cancer, MCC has a markedly decreased 10-year cumulative survival rate (17.7% versus 61.3%, respectively) (Grabowski *et al.*, 2008). Equally dismal, the overall recurrence rate for MCC (40%) is much higher than other skin cancers including melanoma (19%), squamous cell carcinoma (5-9%), or basal cell

carcinoma (1-10%) (McEvoy et al., 2022). Traditionally ineffective systemic treatment of metastatic MCC likely contributes to these particularly dismal statistics.

MCC is managed according to staging with several pan-cancer modalities including surgery, radiation therapy (RT), chemotherapy, and, more recently, immunotherapy. As it stands, no molecular prognostic markers which predict response to therapy or MCC-specific treatments currently exist (Becker *et al.*, 2017). For local or regional disease (stage I-III), excision of the primary tumor and/or draining lymph node followed by adjuvant RT is commonly performed (Villani *et al.*, 2019). Indeed, combination of surgery and RT was shown to significantly increased survival over surgery alone for stage I and II, though no benefit was noted for stage III (Bhatia et al., 2016). These standardized treatments for locoregional MCC can result in long-term control or even resolution of the disease in a few cases, though recurrences are common (Schadendorf *et al.*, 2017). Metastatic MCC (stage IV) has traditionally been treated with chemotherapy. MCC is considered to be highly chemo-sensitive, but responses are not durable, and recurrences often develop within 4-15 months (Schadendorf *et al.*, 2017). Typically a topoisomerase II poison (doxorubicin or etoposide) alone or in combination with a platinum-based DNA crosslinker (carboplatin or cisplatin) is used for treatment, though numerous retrospective studies have not shown significant improvement to survival (Iyer *et al.*, 2016; Tai *et al.*, 2000; Voog *et al.*, 1999). Indeed, some studies found high incidences of death due to chemotherapy-associated toxicity (3.4-7.7%) (Tai *et al.*, 2000; Voog *et al.*, 1999). As a result, chemotherapy for treatment of MCC is considered palliative (Schadendorf *et al.*, 2017).

The advent of immune checkpoint inhibition (ICI) therapy has revolutionized the treatment of metastatic MCC. Briefly, all cells, cancers included, express major histocompatibility complex type I (MHC-I) which present peptides derived from intracellular proteins to immune surveilling T-cells through the T-cell receptor (TCR). The binding of MHC-I to the TCR typically requires association of additional co-factors which regulate T-cell activation and T-cell-dependent anti-tumoral cytotoxicity. One such pair of factors is programmed cell death 1 (PD-1), upregulated on activated T-cells, and programmed cell death ligand 1 (PD-L1), which is often upregulated on cancer cells as well as other cell types. The interaction of PD-1 and PD-L1 suppresses cytotoxic T-cell activity, which is useful for protecting normal tissue from auto-reactive immune responses but is exploited by cancer cells to avoid immune recognition (Liu et al.,

2021). Indeed, increased PD-L1 expression in cancer is associated with worse clinical outcomes (Patel and Kurzrock, 2015). However, MCC is considered a good candidate for ICI through the expression of neoantigens including MCPyV TAg-specific epitopes in MCCP and mutationally-derived epitopes in MCCN (Tanda *et al.*, 2021). With the completion of two landmark studies in 2016 (Kaufman *et al.*, 2016; Nghiem *et al.*, 2016), objective response rates (ORRs) of 31.8-56% were observed in patients who received at least 1 dose of either avelumab (anti-PD-L1) or pembrolizumab (anti-PD-1), respectively. In patients who showed a clinical response, 82-86% were ongoing at the time of study publication (median follow up 33-41 weeks), respectively (Kaufman *et al.*, 2016; Nghiem *et al.*, 2016). Furthermore, when compared to traditional chemotherapy, ICI had dramatically increased overall survival (OS) for patients with metastatic MCC (Tanda *et al.*, 2021). Indeed, MCC is one of the most responsive solid tumors to immunotherapy, with only cutaneous squamous cell carcinoma patients having slightly higher response rates (~52%) (Arora *et al.*, 2019). Despite these promising results, ~50% of metastatic MCC patients do not respond to ICI (Kaufman *et al.*, 2016; Nghiem *et al.*, 2016). Since effective alternatives to ICI do not exist for metastatic MCC, these patients represent a vulnerable population in need of alternative therapeutics.

IMPDH as a druggable target in MCC

Recent efforts from our laboratory have used CRISPR/Cas9-based genetic screening of an MCCP cell line to identify genes essential for survival (Cheng *et al.*, 2017; Park *et al.*, 2020). Interestingly, these screens identified inosine monophosphate dehydrogenase 2 (IMPDH2) as a highly essential gene (Figure 1.2). The IMPDH isozymes (IMPDH1/2) are the rate limiting enzymes of *de novo* guanosine monophosphate (GMP) biosynthesis and function to convert inosine monophosphate (IMP) into xanthosine monophosphate (XMP) which is subsequently converted into GMP (Hedstrom, 2009). IMPDH proteins are 84% identical at the amino acid level and are comprised of two major domains: (1) the catalytic domain which contains the active site and (2) two tandem cystathionine- β -synthase domains (CBS; collectively known as a Bateman domain) which are thought to play a role in the allosteric regulation of IMPDH enzymatic activity through differential binding of guanylate and adenylate nucleotides (Buey *et al.*, 2017; Buey *et al.*, 2015; Hedstrom, 2009; Naffouje *et al.*, 2019; Thomas *et al.*,

2012). IMPDH proteins normally organize into homo- or hetero-tetrameric structures (Hedstrom, 2009), though the proportions of each isozyme and the functional consequences of their molar ratio are unknown. Interestingly, in response to guanylate nucleotide depletion (Ji et al., 2006) or adenylate nucleotide addition (Johnson and Kollman, 2020) the IMPDH tetramers can oligomerize into higher-order filamentous structures (“rods”) through interactions involving the Bateman domain (Keppeke et al., 2018). It is hypothesized that these higher-order structures improve IMPDH catalytic activity (Chang et al., 2015; Keppeke *et al.*, 2018), though this remains disputed (Ji *et al.*, 2006). IMPDH proteins have nearly identical substrate binding kinetics (K_m), reaction rates (k_{cat}), and inhibitory constants (K_i) such that no isozyme-specific inhibitors currently exist (Carr et al., 1993; Hedstrom, 2009). A potential higher-specificity IMPDH2 inhibitor was recently described (Liao et al., 2017), but utility for cancer chemotherapy and off-target effects were not assessed. Although kinetically indistinguishable, IMPDH1 is basally expressed in most tissues whereas IMPDH2 tends to be upregulated during proliferation and in malignancies (Hedstrom, 2009). This upregulation of IMPDH2 in malignant tissue has made IMPDH the focus for strong therapeutic development efforts for decades, though a clinically effective formulation has yet to be identified (Naffouje *et al.*, 2019).

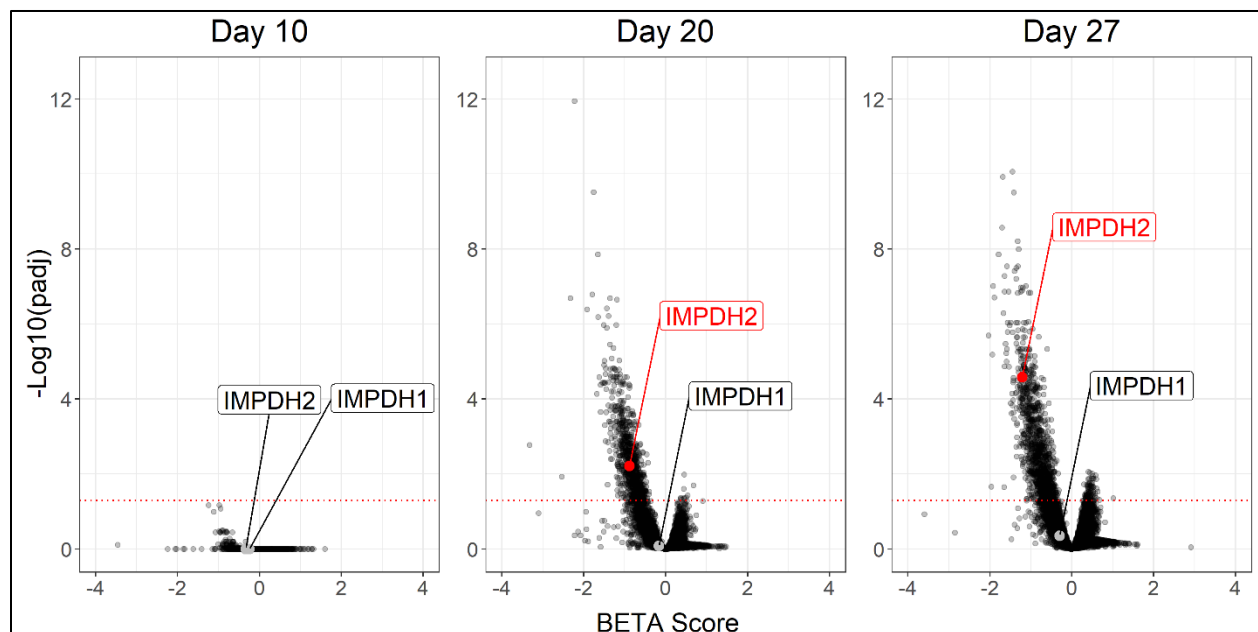


Figure 1.2. CRISPR/Cas9 genetic screens in an MCCP cell line. Data is from genetic screens performed by Park et al and Cheng et al, with IMPDH isozymes highlighted. Data represents Input versus DMSO or untreated at the specified timepoints. “BETA Score” represents $\log_2$ -fold change in sgRNA counts from input to final conditions. Red dotted line and color represent $padj$ value < 0.05, grey indicates $padj$ > 0.05.

The main consequence of IMPDH inhibition in cells is the depletion of guanylate nucleotide levels (Hedstrom, 2009). This depletion has pleiotropic consequences, of which suppression of DNA or RNA synthesis concordant with p53 accumulation and loss of proliferation are typically observed (Huang *et al.*, 2018; Kofuji *et al.*, 2019; Sun *et al.*, 2008; Valvezan *et al.*, 2017). Despite these common observations, a thorough model describing the order of DNA and RNA synthesis loss and the relative importance of each for the anti-proliferative effects of IMPDH inhibition are contested. In addition, whether the effects of IMPDH inhibition are cytostatic or cytotoxic and if these effects depend on p53 activity is unclear.

Several reports in glioblastoma (GBM) (Kofuji *et al.*, 2019) and small-cell lung cancer (SCLC) (Huang *et al.*, 2018) posited that inhibition of IMPDH disproportionately depletes guanylate nucleotides used for nascent RNA synthesis to suppress proliferation. Indeed, radioactively-labeled guanylate nucleotides were shown to be preferentially incorporated into rRNA and tRNA as opposed to mRNA, though incorporation into DNA was not assessed (Kofuji *et al.*, 2019). Furthermore, RT-qPCR analysis of pre-rRNA transcripts showed depletion in GBM and SCLC in response to IMPDH inhibition, while pre-mRNA or mRNA transcripts were unaffected (Huang *et al.*, 2018; Kofuji *et al.*, 2019). It has been hypothesized that inhibition of nascent rRNA synthesis induces the separation and nuclear dispersion of nucleolar protein-RNA complexes in a process termed nucleolar stress (Yang *et al.*, 2018). These “freed” nucleolar proteins have been shown to bind and inhibit the activity of MDM2, leading to activation of p53-dependent gene transactivation and enforcement of cell-cycle arrest or apoptosis (Yang *et al.*, 2018). In agreement with this model, the physical shrinking of nucleoli, dispersion of nucleolar proteins, and accumulation of p53 was observed in GBM cells upon IMPDH inhibition (Kofuji *et al.*, 2019). These findings led the authors to assert that the anti-proliferative of IMPDH inhibition were due, at least in part, to nucleolar stress. However, both GBM and SCLC nearly exclusively retain mutant copies of *TP53* (George *et al.*, 2015; Kandoth *et al.*, 2013) and as such the mechanism by which the anti-proliferative effects of RNA depletion is enforced remain unclear. Furthermore, it should be noted that the cytotoxic effects of IMPDH inhibition were either not assessed (Kofuji *et al.*, 2019) or only marginally increased the proportion of apoptotic cells (~5%; (Huang *et al.*, 2018)).

In contrast to these studies, a separate report in mammalian target of rapamycin (mTORC1)-driven mouse and human cancer cell lines suggested that the main anti-proliferative effects of IMPDH

inhibition were enforced through the depletion of deoxyguanylate nucleotides and subsequent induction of DNA damage (Valvezan *et al.*, 2017). Indeed, upon IMPDH inhibition the authors observed the accumulation of cells in S phase, indicative of a block to DNA replication, in addition to the induction of dsDNA breaks (DSBs) (Valvezan *et al.*, 2017). Furthermore, markers of DSBs and apoptosis were observed *in vitro* and *in vivo*, indicating that IMPDH inhibition and subsequent DNA damage were cytotoxic (Valvezan *et al.*, 2017). Interestingly, co-treatment with RNA polymerase I (mainly responsible for rRNA synthesis) and IMPDH inhibitors relieved markers of DNA damage and restored guanylate nucleotide levels (Valvezan *et al.*, 2017). These findings suggested a model by which nascently generated guanylate nucleotides are preferentially used for rRNA synthesis, in agreement with the other work (Huang *et al.*, 2018; Kofuji *et al.*, 2019). However, despite critical involvement in the response to DNA damage and IMPDH inhibition, neither p53 levels nor activation were assessed (Valvezan *et al.*, 2017) and, moreover, this work was mostly performed in *TP53*-mutant cells (Valvezan *et al.*, 2017).

Several major limitations and inconsistencies in previous studies (Huang *et al.*, 2018; Kofuji *et al.*, 2019; Valvezan *et al.*, 2017) have hampered the convergence of a unified model for the mechanistic consequences of IMPDH inhibition in cancer. One such limitation is the lack of concurrent nucleotide labeling experiments and, as such, the relative timing in suppression of DNA or RNA synthesis upon IMPDH inhibition is not established. It is likely that, depending on the timeframe analyzed, inhibition of IMPDH will eventually suppresses both DNA and RNA synthesis. However, it is currently unclear whether deoxyguanylate or guanylate nucleotides, required for DNA or RNA synthesis, respectively, are initially limiting. Retrospective analysis of nucleotide concentrations in transformed cells found levels of guanosine triphosphate (GTP) ($473 \pm 214 \mu\text{M}$) to be highly enriched (65.7-fold) over that of deoxyguanosine triphosphate (dGTP) ($7.2 \pm 4.4 \mu\text{M}$), indicating that nascent DNA synthesis may initially be limiting upon IMPDH inhibition (Traut, 1994). Another limitation is the lack of consideration for the role of p53 in promoting sensitivity to IMPDH inhibitors. Wildtype p53 activity has long been associated with increased susceptibility to chemotherapeutic regimens (Ferreira *et al.*, 1999; Lu and El-Deiry, 2009), though it is currently unclear if *TP53*-status confers enhanced sensitivity to IMPDH inhibitors. Furthermore, conflicting reports on whether the anti-proliferative effects of IMPDH inhibition are mainly cytostatic or cytotoxic and the factors or pathways required to drive these outcomes are unknown. As a

result, although all reports agree that IMPDH inhibition suppresses cellular proliferation the exact molecular mechanism driving these effects requires further refinement.

Exacerbation of DNA replication stress is an exploitable therapeutic opportunity in cancer

A major consequence of deoxynucleotide triphosphate (dNTP) depletion is the induction of replication stress (RS) in cells actively replicating DNA (Zeman and Cimprich, 2014). RS is defined as the impediment of replication fork progression in response to exogenous (nucleotide analogs or antimetabolites) or endogenous (oncogene hyperactivation) stressors (Zeman and Cimprich, 2014). In eukaryotes, DNA replication occurs in two concerted steps: (1) the genomic origins of DNA replication are defined and bound by the pre-replication complex (pre-RC; “licensing”) and (2) additional replication factors are recruited and DNA synthesis is activated, allowing the fully formed replication forks to proceed bidirectionally outwards from the licensed origin (“firing”) (Fragkos et al., 2015). Although all potential origins of replication are “licensed” during the G1 cell-cycle phase, it is thought that only a small proportion (~10%) are activated during a typical S phase (McIntosh and Blow, 2012). Non-activated origins are considered “dormant”, but in response to RS local “dormant” origins can become activated to ensure complete duplication of the genome while distal “dormant” origins are suppressed from “firing” to conserve replicative resources and prevent additional RS (Fragkos *et al.*, 2015; McIntosh and Blow, 2012). Stalling or stopping of replication forks leads to the accumulation of the heterotrimeric replication protein A (RPA) onto single-stranded DNA (ssDNA) near the stalled fork (Zeman and Cimprich, 2014). The 70 (RPA70), 32 (RPA32), and 14 (RPA14) kDa subunits which comprise RPA are present at a higher molar excess than what is required for normal DNA replication (Toledo et al., 2017) and function to protect vulnerable ssDNA from damage during DNA replication (Marechal and Zou, 2015). High levels of ssDNA-bound RPA at stalled replication forks induce the recruitment and activation of the DDK ataxia telangiectasia and Rad3-related (ATR). Active ATR subsequently phosphorylates checkpoint protein 1 (CHK1), which together cooperate to enforce the intra-S phase or ATR-CHK1 checkpoint (Saldivar et al., 2017). The intra-S checkpoint functions to mitigate and repair stalled or damaged replication forks by broadly suppressing distal “dormant” origin firing, stabilizing stalled replication forks, and promoting fork repair and restart through several mechanisms (Saldivar *et al.*, 2017). Importantly, CHK1 has been shown

to phosphorylate and inhibit the cell division cycle 25 A/B/C (CDC25A/B/C) phosphatases critical for removing inhibitory phosphorylation of cyclin-dependent kinases 1 (CDK1) and cyclin-dependent kinase 2 (CDK2) (Saldivar *et al.*, 2017). CDK1 activity is essential for promoting processes required for efficient mitosis, including commitment to mitotic entry, while CDK2 is important for phosphorylation and inhibition of RB1 to de-repress E2F1/2/3-mediated gene expression and facilitate the transition from the G1 to S cell-cycle phases (Malumbres, 2014; Malumbres and Barbacid, 2005). In addition, CDK-dependent phosphorylation also controls the binding of cell division cycle 45 (CDC45) to the pre-RC, an event that is essential for origin “firing” (Saldivar *et al.*, 2017). As a result, the ATR-CHK1 checkpoint functions to promote a cellular environment conducive for efficient resolution of RS.

Inhibition of the ATR-CHK1 checkpoint in the presence of RS has dire consequences for the replicating cell. Treatment with an ATR inhibitor in the context of dNTP depletion has been shown to induce the hyper-accumulation of RPA onto ssDNA through unchecked “dormant” origin firing, to the extent that RPA levels became limiting in the cell (Toledo *et al.*, 2013). This RPA exhaustion (RE) was shown induce widespread exposure of unprotected ssDNA to severe and irreparable DNA damage through a terminal process termed replication catastrophe (RC) (Toledo *et al.*, 2013). Indeed, disruption of the ATR-CHK1 checkpoint through inhibition of ATR has been shown to improve the efficacy of numerous chemotherapeutic agents (Fokas *et al.*, 2012; Hall *et al.*, 2014; Wallez *et al.*, 2018) and is under heavy clinical investigation in combination with traditional chemotherapeutic regimens (26 clinical trials are recruiting or active as of June 2022). Inhibition of CHK1 has also been assessed as a monotherapy or in combination with traditional chemotherapeutics, but progress has been stymied by dose limiting toxicities (Qiu *et al.*, 2018). Since one of the reported consequences of IMPDH inhibition is the depletion of deoxyguanylate nucleotides, it is possible that IMPDH and ATR inhibitors may interact to synergistically induce RC for therapeutic benefit. However, as it stands, the combinatorial use of IMPDH and ATR inhibitors for cancer therapy have not been studied.

Heterogenous NE gene expression is a prognostic and therapeutic opportunity in MCC

Although MCC is generally considered a uniform NE cancer, careful reassessment of prior expressional analyses has revealed a more nuanced expressional pattern of NE marker genes inter- and

intratumorally. Indeed, variable tumor-level positivity for expression of KRT20 (76-100%) and NEFL/M/H (63-92%) has been observed in MCC (Bobos *et al.*, 2006). In addition, other work has observed MCC tumors with KRT20 staining present only within a subset of cells (10-30%), with no evidence of co-incidence with other cancers (Chan *et al.*, 1997). Furthermore, the presence of SOX2 and ATOH1 expression was observed to vary between individual cells in MCC tumors (50-70%, respectively) (Gambichler *et al.*, 2017; Laga *et al.*, 2010). However, MCC tumors completely negative for expression of ATOH1 or SOX2 have not been reported (Gambichler *et al.*, 2017; Laga *et al.*, 2010). As such, despite numerous reports asserting MCC as a broadly homogenous cancer, subpopulations with heterogenous NE gene expression appear to exist.

The presence of NE and non-NE subpopulations is a common phenotype observed in essentially all NE cancers. Indeed, admixtures of NE and non-NE cells within tumors in addition to “pure” NE and non-NE tumors have been reported in typically NE gastrointestinal, liver, pancreatic, and lung cancers (Frizziero *et al.*, 2020; La Rosa *et al.*, 2016; Volante *et al.*, 2006). However, the mechanism which drives this phenotypic heterogeneity in NE gene expression is currently unclear. Recent evidence in the prototypic NE cancer, SCLC, has suggested that plasticity between NE and non-NE subtypes is driven by the induction of specific transcription factors (Ireland *et al.*, 2020). Indeed, transcription factor-defined NE and non-NE subtypes are well established in SCLC (Rudin *et al.*, 2019). Although the specific drivers of heterogeneity in MCC are unknown, assessment of the relatively highly characterized SCLC NE-defined subtypes may supply insights into the machinations of NE expressional variability in MCC.

SCLC can be divided into several NE-based subtypes each driven by expression of a dominant transcription factor. Indeed, expression of achaete-scute homolog 1 (ASCL1), neurogenic differentiation factor 1 (NEUROD1), POU class 2 homeobox 3 (POU2F3), or Yes1-associated transcriptional regulator 1 (YAP1) each define molecular subtypes of SCLC (Rudin *et al.*, 2019). More broadly, expression of ASCL1 and NEUROD1 define the NE SCLC subtype, concordant with expression of NE markers such as CHGA or SYP (Rudin *et al.*, 2019). On the other hand, POU2F3 and YAP1 define the non-NE subtype, characterized by lack of expression of NE markers (Rudin *et al.*, 2019). Interestingly, ASCL1 and ATOH1 are homologous pro-neural basic helix-loop-helix transcription factors that are normally essential for neuronal specification (Powell and Jarman, 2008), but also required for survival of ASCL1-hi SCLC and

MCC, respectively (Becker *et al.*, 2017; Rudin *et al.*, 2019). Furthermore, induction of MYC proto-oncogene (MYC) in a murine model of ASCL1-hi SCLC was shown to drive a phenotypic shift toward the YAP1-hi SCLC transcriptional program (Ireland *et al.*, 2020). These findings indicated the possibility of MYC-driven phenotypic changes in NE cancer. Interestingly, “variant” cell lines have been described for MCC which have minimal or lack expression of NE genes commonly present in the “classical” NE MCC cell lines (Daily *et al.*, 2015). Furthermore, these “variant” cell lines express MYC, which is otherwise absent in the “classical” NE MCC cell lines (Berrios *et al.*, 2016; Cheng *et al.*, 2017; Daily *et al.*, 2015). As a result, the usefulness of these cells as an accurate model for MCC have been contested. However, when taken in the context of SCLC NE subtypes, it is instead reasonable that these “variant” MCC cell lines could represent outgrowths of non-NE cells from MCC tumors. In this context, disruption of the ATOH1-driven transcriptional program, potentially by aberrant MYC expression, may lead to accumulation of non-NE MCC cells and, eventually, fully non-NE MCC cancer. Although this model could unify the expressional and phenotypic data from MCC tumors and “variant” cell lines, it remains to be assessed. Furthermore, whether these non-NE “variant” MCC cell lines are driven by ectopic MYC expression or express YAP1 is unknown.

In line with the separation of SCLC into NE and non-NE subtypes, expression of YAP1 has recently been suggested as a subtype-defining marker more broadly in cancer. Indeed, it was suggested that all solid cancers could be divided into either YAP1-on or YAP1-off subgroups (Pearson *et al.*, 2021). Strikingly, these authors found YAP-off cancers to be nearly exclusively NE in origin (Pearson *et al.*, 2021). Furthermore, overexpression of YAP1 in YAP1-off cell lines suppressed proliferation, an effect mirrored by knockout of YAP1 or WWTR1 in YAP1-on cell lines (Pearson *et al.*, 2021). As such, these findings indicated an incompatibility of YAP1-driven transcriptional programs in otherwise YAP1-off cancers. Furthermore, it is possible that the variability in NE gene expression observed in MCC is correlated with the transition to a YAP1-hi state as is observed in SCLC and the YAP1-on solid tumors. In any case, YAP1 appears to be a dominant marker of non-NE differentiation pan-cancer.

In line with its apparent critical importance in defining subtypes pan-cancer, YAP1 functions as part of the evolutionarily conserved Hippo signaling pathway which directs cellular responses to broad stimuli. The core Hippo pathway is composed of the mammalian Ste20-like kinases 1/2 (MST1/2) and

large tumor suppressor 1/2 (LATS1/2) kinases in addition to the adaptor proteins MOB kinase activator 1A/1B (MOB1A/B) and Salvador homologue 1 (SAV1) (Harvey *et al.*, 2013). Several intrinsic and extrinsic stimuli can lead to the activation of MST1/2 and LATS1/2 including cell-cell contact, cell polarity, extracellular matrix (ECM) interactions, liquid shear stress, hormones or growth factors, and other cellular stressors (Dong *et al.*, 2007). Upon activation, MST1/2 phosphorylates and activates LATS1/2 which subsequently phosphorylates the transcription factors YAP1 and WW-domain containing transcriptional regulator 1 (WWTR1) (Harvey *et al.*, 2013). Phosphorylation of YAP1 and WWTR1 is inhibitory and prevents downstream YAP1- and WWTR1-dependent transcriptional activity through phospho-dependent cytosolic sequestration and ubiquitin-mediated degradation (Dong *et al.*, 2007). When unphosphorylated, YAP1 and WWTR1 can associate with their obligate DNA-binding partner TEA domain transcription factors 1-4 (TEAD1-4) and transactivate expression of diverse transcriptional programs involved in cellular proliferation, survival, motility, stemness, and differentiation (Ma *et al.*, 2019). Due to the importance of these processes in oncogenesis, the Hippo pathway is commonly dysregulated in cancer (Harvey *et al.*, 2013).

Recently, the expression of YAP1 or NE genes has been shown to have prognostic and therapeutic relevance in cancer. Indeed, patients with NE (ASLC1 or NEUROD1) SCLC have been described to have worse OS and progression-free survival (PFS) than those with non-NE subtypes (POU2F3 or YAP1) (Qi *et al.*, 2022). Of the markers in this analysis, the YAP1 subtype correlated with the best OS and PFS (Qi *et al.*, 2022). However, other reports in separate cancer types found expression of YAP1 to correlate with worse overall prognosis (Garcia *et al.*, 2020; Guo *et al.*, 2019; Zhou *et al.*, 2020). Despite divergent conclusions, all reports agree that YAP1 expression has cancer-specific prognostic value. In addition to prognostic significance, subtype-specific therapeutics are currently under clinical development for targeted treatment of NE and non-NE SCLC (Schwendenwein *et al.*, 2021). Although none of these modalities have received FDA-approval, the exploitation of NE-status for therapeutic intervention remains a promising avenue of research which could be translatable to other NE cancers.

Taken together, expressional analyses of MCC tumors have suggested the potential for previously unrecognized non-NE cellular populations commonly observed in other NE malignancies. However, the transcriptional environment which potentially drives these NE-based phenotypic changes in

MCC is currently undefined. Despite these limitations, a better understanding of the heterogenous expressional environment within MCC tumors could lead to the development of targeted therapeutic modalities and enhanced patient outcomes.

Chapter 2

IMPDH inhibition rapidly induces DNA replication stress in Merkel cell carcinoma

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Author Contributions

Thomas C. Frost: Conceived the project, designed and conducted all experiments unless otherwise noted below, and wrote the manuscript.

Adam C. Wang: Assisted in acquiring and analyzing data for Figure 2.2D and wrote corresponding methods section.

Varsha Ananthapadmanabhan: Assisted in acquiring data and experimental design for Figure 2.2E.

James A. DeCaprio: Supervised the study, assisted in conceptualization, and edited the manuscript.

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Declaration of Interests

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Summary

The rate-limiting isozyme of *de novo* guanosine biosynthesis, *IMPDH2*, was recently identified as an essential gene in Merkel cell carcinoma (MCC) but the consequences of its inhibition are unclear. Through suppression of IMPDH activity, we stratified MCC sensitivity groups based on the depletion of guanylate nucleotides. This sensitivity resulted in cytotoxicity that was suppressed upon genetic or functional loss of p53. We determined that ablation of nascent DNA but not RNA synthesis and the rapid induction of replication stress was the dominant outcome of IMPDH inhibition. Combined treatment with IMPDH and ATR inhibitors increased levels of replicative stress and replication catastrophe independent of p53 status. These findings provide mechanistic understanding and rationale for therapeutic IMPDH targeting in *TP53*-wildtype and -mutant contexts.

Introduction

Merkel cell carcinoma (MCC) is an aggressive neuroendocrine carcinoma of the skin with two genetically distinct subtypes defined by the presence or absence of Merkel cell polyomavirus (MCPyV) (Becker *et al.*, 2017; Feng *et al.*, 2008). Virus-negative MCC (MCCN) is characterized by a high tumor mutational burden due to extensive UV exposure and typically harbors inactivating mutations in the tumor suppressor genes *TP53* and *RB1* (Knepper *et al.*, 2019; Starrett *et al.*, 2020). Polyomavirus-positive MCC (MCCP) features clonal integration of MCPyV DNA into the host genome with persistent expression of the viral oncoproteins small T (ST) and a truncated form of large T antigen (LT). LT binds and inactivates *RB1* to overcome the G1/S cell cycle checkpoint (Cheng *et al.*, 2013), while ST forms a transcriptional activator complex with EP400 and MYCL that induces expression of the p53-targeting E3 ubiquitin ligase MDM2 to suppress p53 levels (Cheng *et al.*, 2017; Park *et al.*, 2019). Therefore, although the *TP53* and *RB1* genes are usually wildtype in MCCP, their function is suppressed by the viral oncoproteins. However, in MCCP cells, p53 can become activated in response to cellular stress, including MDM2 inhibition, to overcome T antigen-mediated repression (Houben *et al.*, 2013; Park *et al.*, 2019).

Despite the profound genetic differences between MCCN and MCCP tumors, clinical treatment of MCC is not stratified based upon subtype. Typically, local disease is surgically excised followed by adjuvant radiotherapy while metastatic MCC has been treated with chemotherapy with rapid emergence of resistance (Becker *et al.*, 2017; Iyer *et al.*, 2016; Tai *et al.*, 2000; Villani *et al.*, 2019; Voog *et al.*, 1999). Recently, the development of anti-PD-1/PD-L1 antibodies for immune checkpoint inhibition (ICI) has shown promise for the treatment of advanced stage MCC (Kaufman *et al.*, 2016; Nghiem *et al.*, 2016; Villani *et al.*, 2019). However, approximately 50% of patients do not respond to ICI (Kaufman *et al.*, 2016; Nghiem *et al.*, 2016) and represent a vulnerable patient population in need of alternative therapy. As a result, identifying additional targetable vulnerabilities in MCC is urgently needed.

A recent genome-wide CRISPR/Cas9 knockout screen in the MCCP MKL-1 cell line identified inosine monophosphate dehydrogenase 2 (*IMPDH2*) as an essential gene (Cheng *et al.*, 2017). *IMPDH1* and *IMPDH2* are the rate limiting isozymes for *de novo* guanosine monophosphate (GMP) biosynthesis that convert inosine monophosphate (IMP) into xanthosine monophosphate (XMP) from which GMP can be generated (Hedstrom, 2009). Newly synthesized guanylate nucleotides can be utilized for DNA and

RNA synthesis and GTP-based signaling, among other functions (Hedstrom, 2009). Though IMPDH1 and IMPDH2 share 84% amino acid identity and have nearly identical kinetic properties *in vitro*, IMPDH2 tends to be specifically upregulated in cancer (Hedstrom, 2009). Since no IMPDH-specific inhibitors that distinguish between IMPDH1 and IMPDH2 are currently available, inhibition of IMPDH suppresses both isozymes (Naffouje *et al.*, 2019). As such, IMPDH inhibition has been shown to deplete guanylate nucleotide levels and to induce cellular stress marked by the activation of p53 (Huang *et al.*, 2008; Kofuji *et al.*, 2019; Sun *et al.*, 2008; Valvezan *et al.*, 2017). However, despite all reports observing a reduction in viability upon IMPDH inhibition, the mechanism by which this occurs has been disputed. Some studies observed a prolonged S-phase and the accumulation of DNA damage as an indication that deoxynucleotide triphosphates (dNTPs) were limiting (Valvezan *et al.*, 2017), while others observed the reduction of nascent pre-RNA synthesis to indicate that nucleotide triphosphate (NTPs) were limiting (Huang *et al.*, 2008; Kofuji *et al.*, 2019). However, most prior studies did not assess the effect of IMPDH inhibition on DNA and RNA synthesis concurrently and it remains unclear which effect is dominant. Notably, metabolic analysis of normal and transformed cells showed baseline concentrations of GTP are substantially higher (65.7-fold) than dGTP (Traut, 1994), supporting the notion that DNA synthesis may be initially limiting in response to suppression of *de novo* guanylate nucleotide biosynthesis.

Depletion of dNTPs can induce DNA replication stress (RS) in a variety of cancers (Zeman and Cimprich, 2014) and is often the basis for chemotherapeutic regimens (Chabner and Roberts, 2005). In response to RS, ataxia telangiectasia and Rad3-related (ATR) is recruited to single-stranded DNA (ssDNA) by interaction with the heterotrimeric replication protein A (RPA), leading to ATR-dependent checkpoint kinase 1 (CHK1) phosphorylation and subsequent activation of the intra-S checkpoint (Saldivar *et al.*, 2017). The ATR-CHK1 checkpoint prevents mitotic entry prior to the completion of DNA replication and inhibits dormant origin firing to preserve genomic integrity (Saldivar *et al.*, 2017; Toledo *et al.*, 2017). Inhibition of ATR in the context of RS was shown to induce the widespread generation of ssDNA with subsequent recruitment of RPA through unchecked dormant origin firing (Toledo *et al.*, 2013). At a critical point, the amount of ssDNA generated can exceed global RPA levels resulting in RPA exhaustion (RE) and the exposure of unprotected ssDNA to extensive damage leading to an end-stage event termed replication catastrophe (RC) (Toledo *et al.*, 2013). Inhibition of ATR was found to enhance

the efficacy of several RS-inducing chemotherapeutic agents (Fokas *et al.*, 2012; Hall *et al.*, 2014; Wallez *et al.*, 2018), though the combination of ATR and IMPDH inhibition has not been assessed.

In the present work, we stratified MCC cell lines according to their sensitivity to IMPDH inhibition and found that this sensitivity depends on functional p53. We determined that reduction in nascent DNA synthesis and generation of RS are the dominant mechanistic outcomes of IMPDH inhibition. Building on these results, we observed that ATR inhibition accentuated the RS induced by IMPDH inhibition leading to RC even in the absence of p53 activity. Taken together, our work identifies IMPDH as a key therapeutic vulnerability in MCC and provides mechanistic insight into the use of IMPDH and ATR inhibitors as combinatorial therapy.

Results

***TP53*-wildtype MCC cell lines are highly sensitive to IMPDH inhibition**

To assess the sensitivity of MCC to IMPDH inhibition, we treated a panel of established MCCC cell lines with increasing doses of the IMPDH inhibitor mycophenolic acid (MPA) and measured cell viability with the CellTiter-Glo assay (Figure 2.1A). Increasing dosages of MPA led to decreased viability in all cell lines tested. Notably, MKL-1, WaGa, and PeTa were each significantly more sensitive than MS-1 cells. MS-1 contains a hemizygous deletion in *TP53* which renders p53 non-functional in contrast to the other MCCC lines which retain wildtype *TP53* (Houben *et al.*, 2013; Park *et al.*, 2019).

We further evaluated the on-target activity of MPA in WaGa and MKL-1 cell lines. Sensitivity to MPA treatment in the CellTiter-Glo assay was recapitulated with direct measurement of viable cell counts (WaGa; Figure 2.2A) and upon treatment with alternative IMPDH inhibitors including AVN-944 (VX-944) and mycophenolate mofetil (MMF, CellCept), a prodrug formulation of MPA (WaGa & MKL-1; Figure 2.2B). Co-treatment of MPA with guanosine rescued viability in a dose-dependent manner in each cell line (Figure 2.1B, 2.2C). Liquid chromatography-mass spectrometry (LC-MS) was performed to determine the effects of MPA on cellular nucleotide pools in MCC (Figure 2.2D). Within 4 hours of MPA treatment, we observed maximal detectable loss (~4-fold) of GMP nucleotides that was sustained for at least 12 hours. This effect directly coincided with a marked increase (~10-fold) in IMP nucleotides at each timepoint. Importantly, concentrations of AMP were unaffected over the course of the experiment. Taken together, these results indicate that sensitivity to IMPDH inhibition in MCC was dependent on the specific depletion of GMP nucleotides.

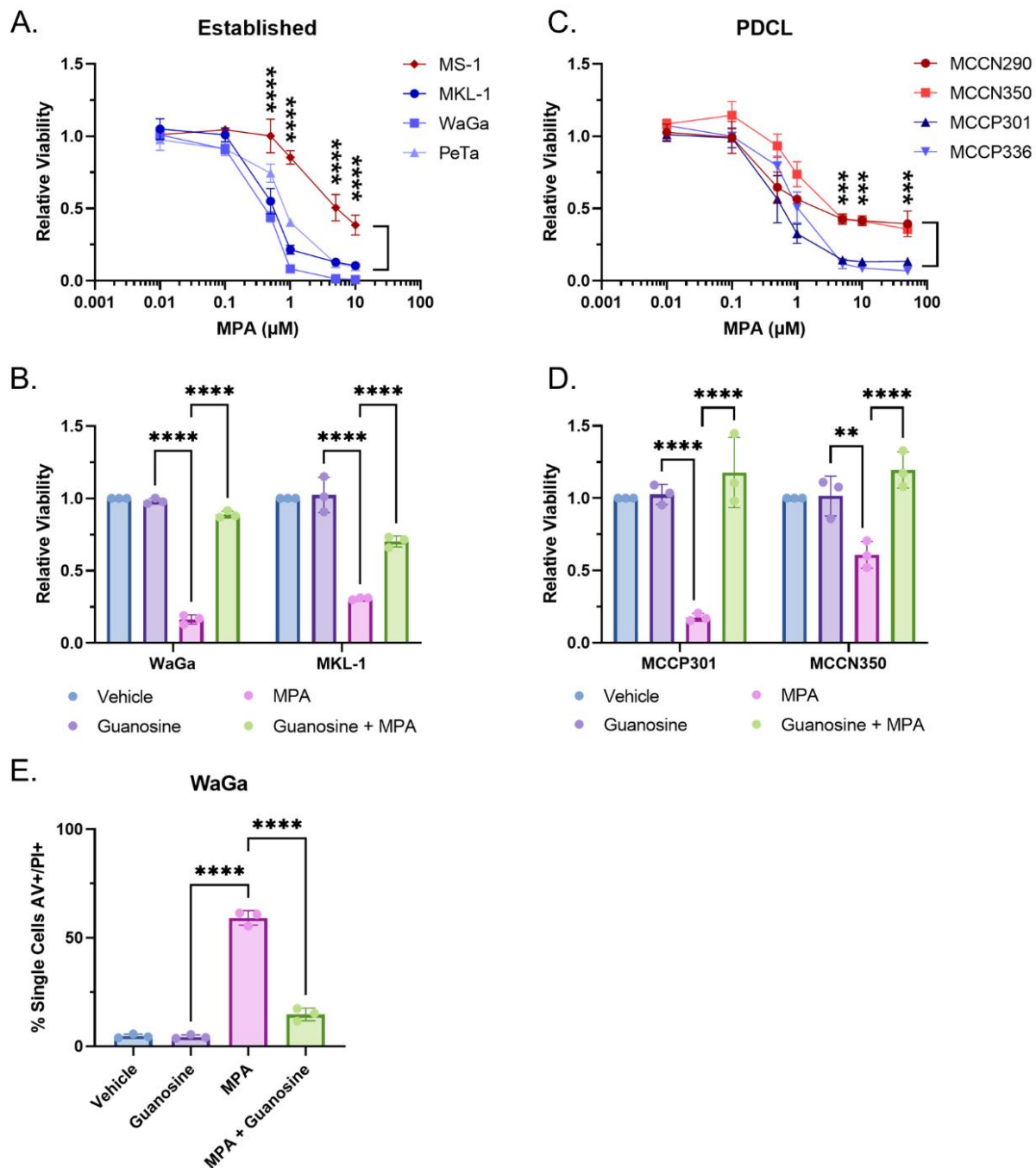


Figure 2.1. *TP53*-wildtype MCC cell lines are highly sensitive to IMPDH inhibition. (A) CellTiter-Glo viability assay of established MCCCP cell lines treated with increasing dosages of MPA for 3 days. Line color identifies p53-wildtype (blues) and -mutant (red) cell lines. Statistics represent lowest significance (highest p) of any p53-wildtype versus -mutant comparisons at the specified dose. $N = 3$; mean $\pm$ SD; 2-way ordinary ANOVA corrected for multiple comparisons via Tukey *post hoc* test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (B) CellTiter-Glo viability assay of representative IMPDH inhibitor-sensitive established MCCCP cell lines treated concurrently with MPA (1 μM) and guanosine (10 μM) for 3 days. $N = 3$; mean $\pm$ SD; 2-way ordinary ANOVA corrected for multiple comparisons via Tukey *post hoc* test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (C) CellTiter-Glo viability assay of PDCLs treated as in

(Figure 2.1, continued)

(A), with an additional dose (50 μ M). Line coloring and statistical test are identical to (A). N = 3; mean $\pm$ SD. (D) CellTiter-Glo viability assay of PDCLs treated concurrently with MPA (5 μ M) and guanosine (1 μ M) for 3 days. N = 3; mean $\pm$ SD; 2-way ordinary ANOVA corrected for multiple comparisons via Tukey *post hoc* test; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. (E) AV/PI staining of WaGa cells treated with MPA (1 μ M) and guanosine (10 μ M) for 2 days. N= 3; mean $\pm$ SD; 1-way ordinary ANOVA corrected for multiple comparisons via Tukey *post hoc* test; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

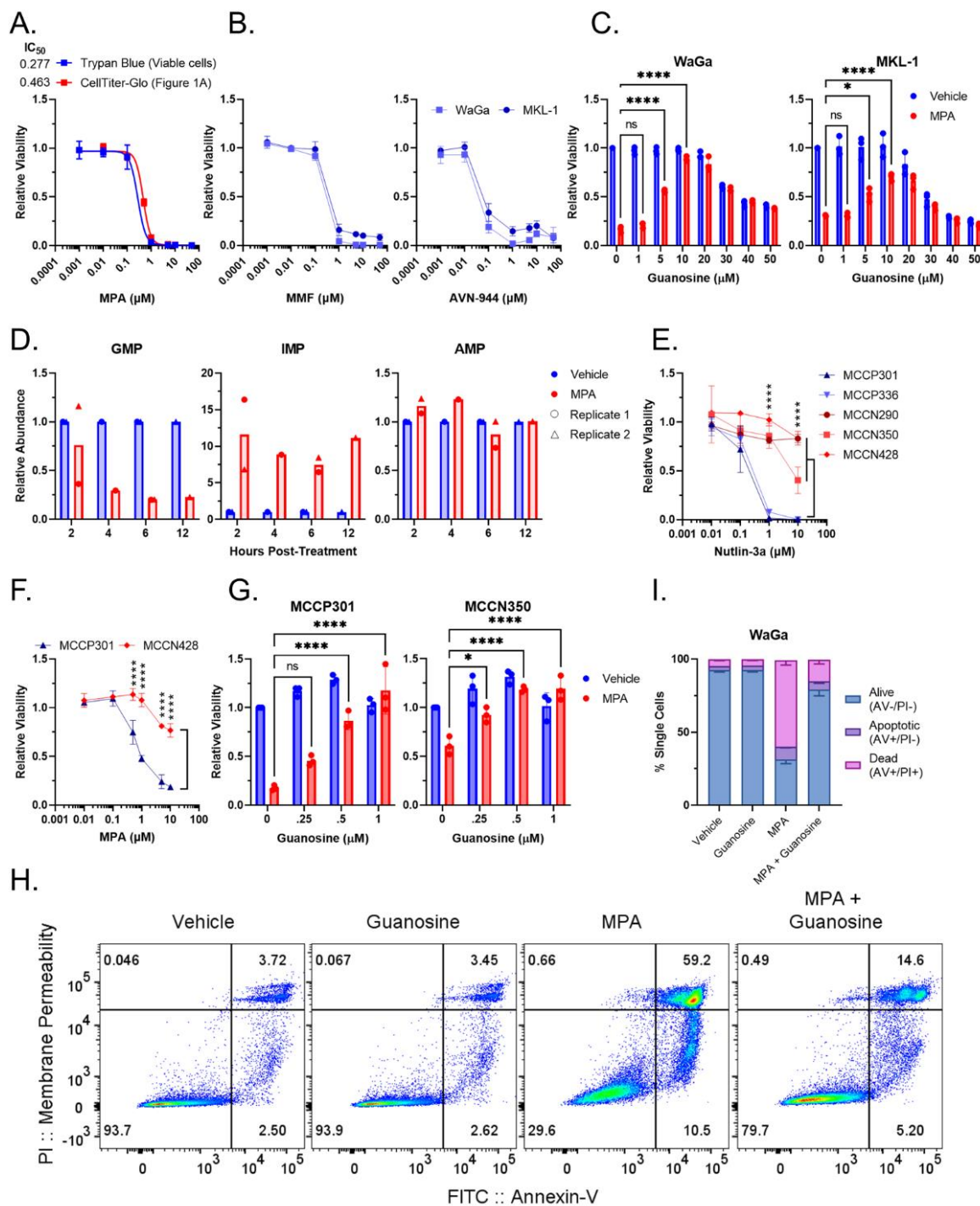


Figure 2.2. Mechanistic validation of IMPDH inhibition in MCC cell lines. (A) Comparison of CellTiter-Glo (from Figure 2.1A) and trypan blue viability assay in WaGa cells treated with increasing dosages of MPA for 3 days. IC₅₀ was calculated for each method. N = 3; mean ± SD. (B) CellTiter-Glo viability assay of WaGa and MKL-1 cells treated with increasing dosages of alternative IMPDH inhibitors MMF and ANV-944 for 3 days. N = 3; mean ± SD. (C) CellTiter-Glo viability assay of WaGa and MKL-1 cells treated concurrently with MPA (1 μM) and increasing dosages of guanosine. N = 3; mean ± SD; 2-way ordinary ANOVA corrected for multiple comparisons via Tukey *post hoc* test; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. (D) LC-MS analysis of key purine nucleotides from WaGa cells treated with MPA (1 μM)

(Figure 2.2, continued)

for up to 12 hours. N = 1 for 4- & 12-hour timepoint, N = 2 for 2- & 6-hour timepoints; bars represent mean, shapes denote specific replicates. (E) CellTiter-Glo viability assay of PDCLs treated for 5 days with increasing dosages of nutlin-3a. Line color identifies p53-wildtype (blues) and -mutant (red) cell lines. Statistics represent lowest significance (highest *p*) of any p53-wildtype verses -mutant comparisons at the specified dose. N = 3; mean $\pm$ SD; 2-way ordinary ANOVA corrected for multiple comparisons via Tukey *post hoc* test; * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001, **** *p* < 0.0001. (F) CellTiter-Glo viability assay of additional PDCL MCCN428 treated with increasing dosages of MPA. MCCP301 serves as a sensitivity control. Statistics represent lowest significance (highest *p*) of comparisons at the specified dose. N = 3; mean $\pm$ SD; 2-way ordinary ANOVA corrected for multiple comparisons via Tukey *post hoc* test; * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001, **** *p* < 0.0001. (G) CellTiter-Glo viability assay of MCCP301 and MCCN350 PDCLs treated concurrently with MPA (5 μ M) and increasing dosages of guanosine. N = 3; mean $\pm$ SD; 2-way ordinary ANOVA corrected for multiple comparisons via Tukey *post hoc* test; * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001, **** *p* < 0.0001. (H) Flow cytometry diagram of AV/PI staining in WaGa cells treated with MPA (1 μ M) and guanosine (10 μ M) for 2 days. Quantification of these data are in Figure 1E. Representative of 3 independent experiments. (I) Percentage of single cells that were alive, apoptotic, or dead from (I) and Figure 1E. N = 3; mean $\pm$ SD.

To extend these results, we assessed sensitivity to IMPDH inhibition in a panel of newly generated MCC patient-derived cell lines (PDCL) including both MCCP (MCCP301 and MCCP336) and MCCN (MCCN290, MCCN350, and MCCN428) subtypes. To determine functional p53 status, the PDCLs were treated with the MDM2 inhibitor nutlin-3a (Figure 2.2E). Each MCCP cell line was highly sensitive, while the MCCN were resistant to MDM2 inhibition. Treatment with MPA dose-dependently decreased viability in all the PDCLs, irrespective of viral status (Figure 2.1C, 2.2F). PDCLs with functional p53 activity (MCCP301 and MCCP336) reached significantly higher maximal inhibition compared to those with non-functional p53 (MCCN290, MCCN350, and MCCN428). The viability defect was rescued with supplementation of guanosine in a dose-dependent manner regardless of viral and p53 status in representative cell lines from each PDCL group (Figure 2.1D, 2.2G).

To characterize the viability loss observed after MPA treatment in an established MCCP cell line with functional p53, we performed annexin-V (AV) and propidium iodide (PI) staining in WaGa cells (Figure 2.1E, 2.2H). Strikingly, within two days of treatment we observed a significant population of dead cells (~60%, AV+/PI+), that was rescued with guanosine co-treatment. A modest accumulation of apoptotic cells (AV+/PI-) was also observed upon MPA treatment relative to vehicle or guanosine alone (Figure 2.2I). Collectively, these findings implicate p53 as a critical cytotoxic effector of IMPDH inhibition.

Functional p53 is required for IMPDH inhibition-induced cytotoxicity in MCC

To determine if p53 was specifically activated in MCC when IMPDH was inhibited, WaGa and MKL-1 cells were treated with MPA and assessed for p53 activation by immunoblot (Figure 2.3A, 2.4A). In both cell lines, MPA induced p53 phosphorylation (phospho-Ser15; pp53) coinciding with the accumulation of total p53 protein. In addition, expression of downstream p53 targets including p21 (*CDKN1A*) and PUMA (*BBC3*) was induced at the protein and mRNA levels (Figure 2.4B). In agreement with AV/PI staining (Figure 2.1E), MPA treatment led to cleavage of caspase-3 (CASP3) and its substrate PARP-1 indicating apoptosis was induced (Elmore, 2007). Importantly, markers of p53 activation and apoptosis were reduced or eliminated by guanosine co-treatment.

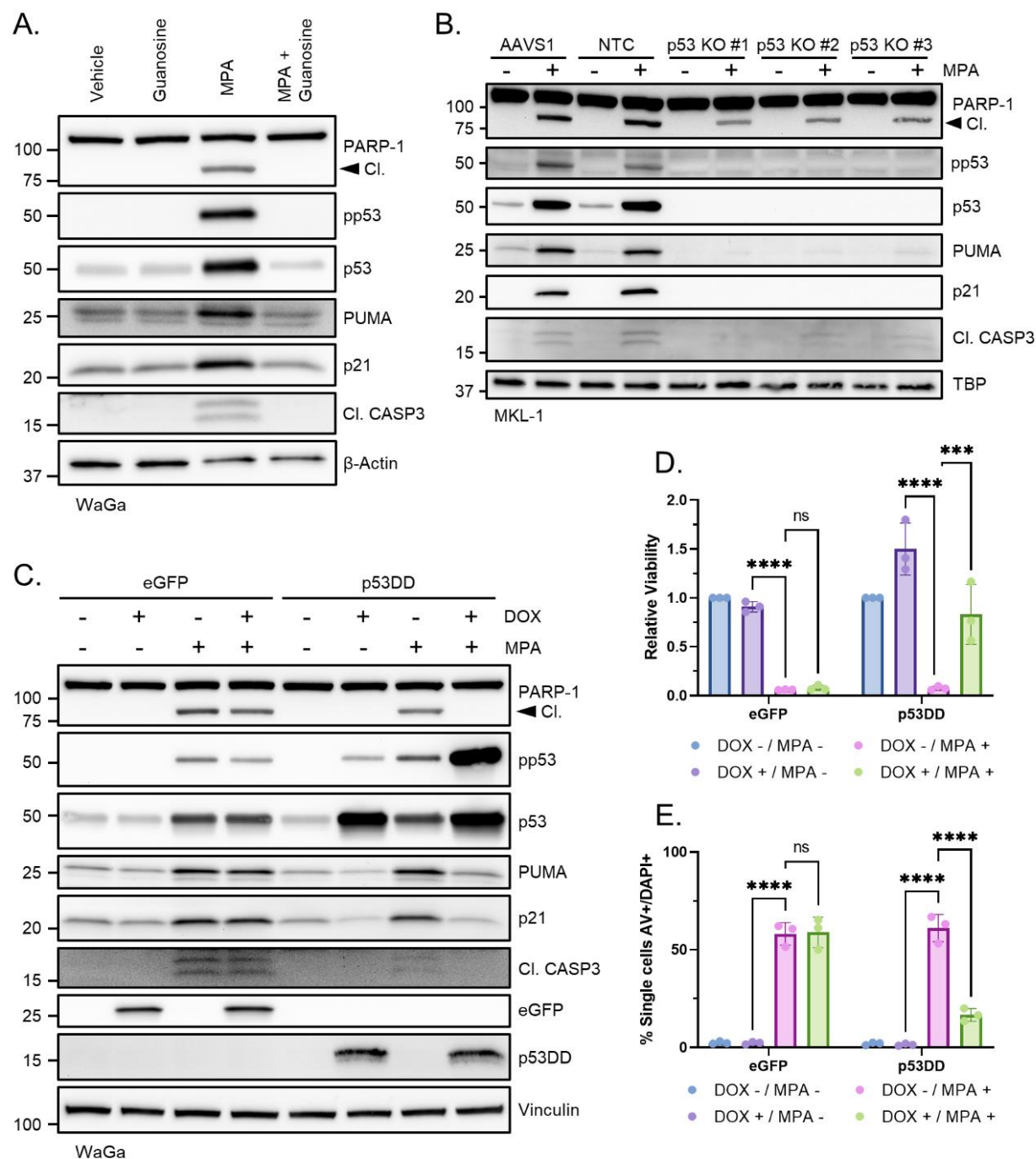


Figure 2.3. Functional p53 is required for IMPDH inhibition-induced cytotoxicity in MCC. (A) Immunoblot of WaGa cells treated with MPA (1 μ M) and guanosine (10 μ M) for 24 hours. Representative of 3 independent experiments. (B) Immunoblot of single-cell cloned p53 knockout MKL-1 cells treated with MPA (1 μ M) for 3 days. Representative of 3 independent experiments. (C) Immunoblot of polyclonal WaGa cells with inducible p53DD or eGFP pretreated with DOX (1 μ g/mL) for 24 hours followed by MPA (1 μ M) for an additional 24 hours. Representative of 3 independent experiments. (D) CellTiter-Glo viability assay of WaGa cells treated as in (C) for 3 days. N = 3; mean $\pm$ SD; 2-way ordinary ANOVA corrected for multiple comparisons via Tukey *post hoc* test; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. (E) AV/DAPI staining of WaGa cells treated as in (C) for 2 days. N = 3; mean $\pm$ SD; 2-way ordinary ANOVA

(Figure 2.3, continued)

corrected for multiple comparisons via Tukey *post hoc* test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

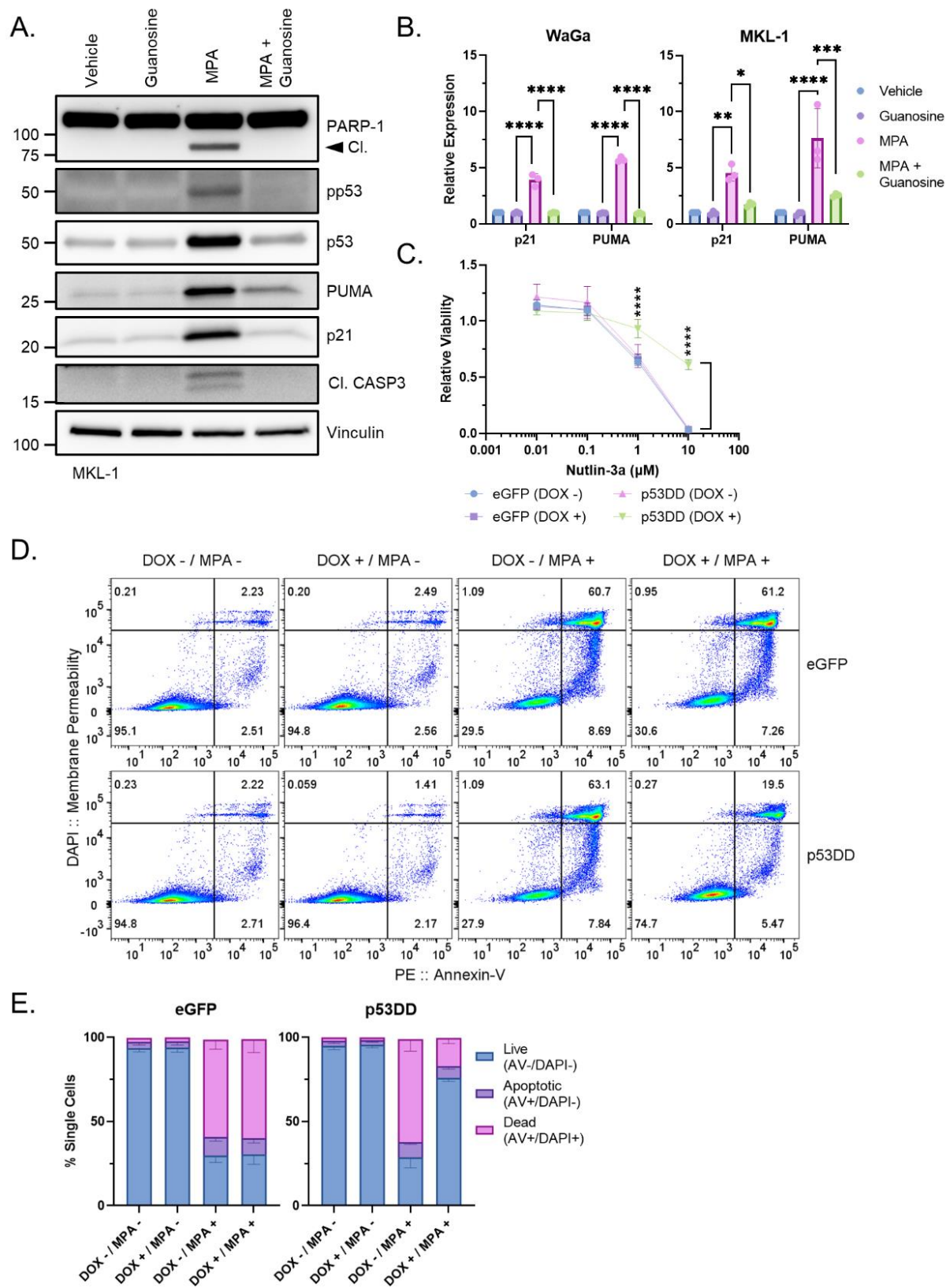


Figure 2.4. Additional assessment of p53-dependent cytotoxicity upon IMPDH inhibition. (A) Immunoblot of MKL-1 cells treated with MPA (1 μ M) and guanosine (10 μ M) for 3 days. Representative of 3 independent experiments. (B) RT-qPCR analysis of p53-dependent gene activation in WaGa and MKL-1 cells treated with MPA (1 μ M) and guanosine (10 μ M) for 1 or 3 days, respectively. Genes were normalized to the geometric mean of β -actin and β -2-microglobulin via the $\Delta\Delta$ Ct method. N = 3; mean $\pm$ SD; 2-way ordinary ANOVA corrected for multiple comparisons via Tukey *post hoc* test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (C) CellTiter-Glo viability assay of WaGa cells with inducible p53DD or eGFP pre-treated with DOX (1 μ g/mL) for 24 hours followed by increasing nutlin-3a dosages for 3 days. Statistics represent lowest significance (highest p) of comparisons at the specified dose. N = 3; mean $\pm$ SD; 2-way ordinary ANOVA corrected for multiple comparisons via Tukey *post hoc* test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (D) Flow cytometry diagram of AV/DAPI staining from Figure 2E. (E) Percentage of single cells that were alive, apoptotic, or dead from Figure 2E. N = 3; mean $\pm$ SD.

Since p53 was activated upon MPA treatment, we sought to determine if p53 was required for MPA-induced cytotoxicity. To this end, we generated three single cell clones of MKL-1 with CRISPR/Cas9-mediated *TP53* knockout along with controls including a non-targeting sgRNA (NTC) and a sgRNA targeting the safe harbor adeno-associated virus integration site 1 (AAVS1 or *PPP1R12C*) (Mali et al., 2013). Upon MPA treatment, all three *TP53* knockout clones had decreased levels of cleaved CASP3 and PARP-1 relative to either NTC or AAVS1 controls (Figure 2.3B). Total and phosphorylated p53 were not detected in the *TP53* knockout cells nor were levels of p21 or PUMA changed in response to MPA treatment.

To confirm the role of p53 in the response to IMPDH inhibition, we transduced WaGa cells with doxycycline (DOX)-inducible dominant negative p53DD (Bowman et al., 1996) or enhanced GFP (eGFP). Functional p53 disruption in p53DD-expressing cells was confirmed by resistance to nutlin-3a (Figure 2.4C). To assess the effect of functional p53 loss on sensitivity to IMPDH inhibition, we induced expression of p53DD or eGFP followed by MPA treatment and harvested protein for immunoblot analysis (Figure 2.3C). As expected, accumulation of endogenous p53 was observed upon induction of p53DD. This was likely due to the lack of MDM2-interacting residues (18-23aa) in p53DD, allowing for enhanced hetero-tetramerization and stabilization of endogenous p53 (Chene, 2003; Katz et al., 2018; Midgley and Lane, 1997). Induction of p53DD prevented increases in levels of p21 and PUMA protein upon MPA treatment in addition to reducing levels of CASP3 and PARP-1 cleavage. We assessed cellular viability after MPA and DOX treatment with the CellTiter-Glo assay (Figure 2.3D). Expression of p53DD partially rescued cell viability in response to MPA treatment. We further characterized the effect of functional p53 disruption on MPA-mediated apoptosis by AV/DAPI staining (Figure 2.3E, 2.4D). Induction of eGFP did not affect the proportion of dead cells (~60%, AV+/DAPI+) upon MPA treatment. However, expression of p53DD significantly decreased the population of dead cells (~15%) when compared to eGFP or the uninduced controls. A minimal increase in apoptotic cells (AV+/DAPI-) was observed upon MPA treatment (Figure 2.4E). Taken together, these data indicate that functional p53 is required for the cytotoxic response to IMPDH inhibition in MCC cells.

IMPDH inhibition rapidly abolishes dNTP incorporation into nascent DNA

Despite the profound p53-dependent cytotoxicity observed upon IMPDH inhibition, the exact mechanism for p53 accumulation and activation was unclear. Loss of GMP nucleotides has been reported to limit nascent RNA (Huang *et al.*, 2008; Kofuji *et al.*, 2019) and DNA (Valvezan *et al.*, 2017) synthesis leading to p53 activation, but it was not known what was limiting in MCC. To assess nascent DNA and RNA synthesis, we treated WaGa cells with MPA and labeled DNA with 5-ethynyl-2'-deoxyuridine (EdU; Figure 2.5A) or RNA with 5-ethynyl uridine (5EU; Figure 2.5B). Incorporation into DNA and RNA was detected by click-chemistry addition of fluorescent dyes followed by flow cytometry. Specific labeling of nascent DNA or RNA was confirmed by treatment with the DNA synthesis inhibitors aphidicolin or hydroxyurea (HU) and the RNA synthesis inhibitor actinomycin-D (ActD). Treatment with aphidicolin or HU completely ablated DNA synthesis (Figure 2.5A, 2.5C), while not significantly perturbing the percentage of nascent RNA-positive cells (Figure 2.5B, 2.5D) or the mean fluorescent intensity (MFI) of NTP incorporation (Figure 2.6A). ActD is a DNA-intercalating agent and induced partial suppression of dNTP incorporation, though not to the extent of either aphidicolin or HU (Figure 2.5C). However, ActD prevented nascent RNA synthesis (Figure 2.5D, 2.6A).

Strikingly, the ablation of all nascent dNTP incorporation occurred within 4 hours of MPA treatment and was sustained for 24 hours (Figure 2.5A, 2.5C). This timeline coincided with the maximal depletion of GMP nucleotides detected by LC-MS (Figure 2.6D). By contrast, MPA had no significant effect on the proportion of cells positive for nascent RNA synthesis (Figure 2.5D) or the MFI of cells with incorporated 5EU (Figure 2.6A). These results strongly implicate the suppression of nascent DNA synthesis as the dominant effect of IMPDH inhibition.

To validate the on-target effects of MPA on nascent DNA synthesis, we co-treated WaGa cells with MPA and guanosine followed by EdU labeling (Figure 2.5E, 2.5F). MPA treatment rapidly reduced dNTP incorporation, an effect that was completely rescuable with supplementation of guanosine. To extend this observation, we performed EdU labeling in the PDCLs MCCP301 and MCCN428 (Figure 2.6B, 2.6C). Loss of dNTP incorporation upon MPA treatment was observed in both PDCLs, an effect that was rescued by guanosine supplementation. These results indicate that the differences in sensitivity may reflect the stress response induced by IMPDH inhibition.

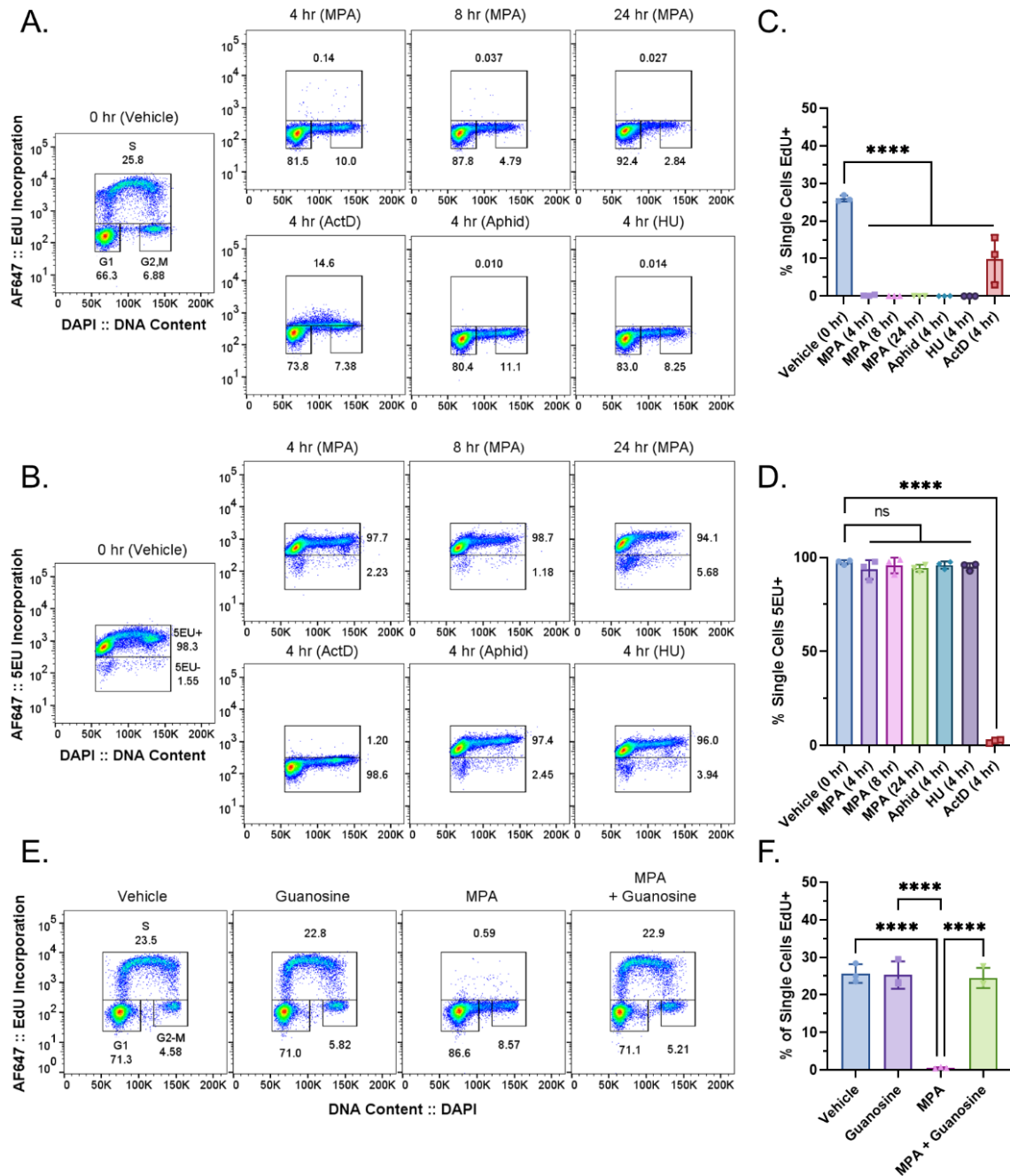


Figure 2.5. IMPDH inhibition rapidly abolishes dNTP incorporation into nascent DNA. (A) Flow cytometry analysis of WaGa cells treated with MPA (1 μ M), ActD (1 μ M), aphidicolin (10 μ M) and HU (1 mM) for up to 24 hours, pulsed with EdU (10 μ M) in the final hour. Images generated in FlowJo. Representative of 3 independent experiments. (B) Flow cytometry analysis of WaGa cells treated as in (A) but pulsed with 5EU (1 mM) in the final hour. Images generated in FlowJo. Representative of 3 independent experiments. (C) Quantification of single cells positive for EdU from (A). N = 3; mean $\pm$ SD; 1-way ordinary ANOVA corrected for multiple comparisons via Tukey *post hoc* test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (D) Quantification of single cells positive for 5EU from (B). N = 3; mean $\pm$ SD; 1-way ordinary ANOVA corrected for multiple comparisons via Tukey *post hoc* test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (E) Flow cytometry analysis of WaGa cells treated concurrently with

(Figure 2.5, continued)

MPA (1 μ M) and guanosine (10 μ M) for 4 hours, pulsed with EdU (10 μ M) in the final hour. Images generated in FlowJo. Representative of 3 independent experiments. (F) Quantification of single cells positive for EdU from (E). N = 3; mean $\pm$ SD; 1-way ordinary ANOVA corrected for multiple comparisons via Tukey *post hoc* test; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

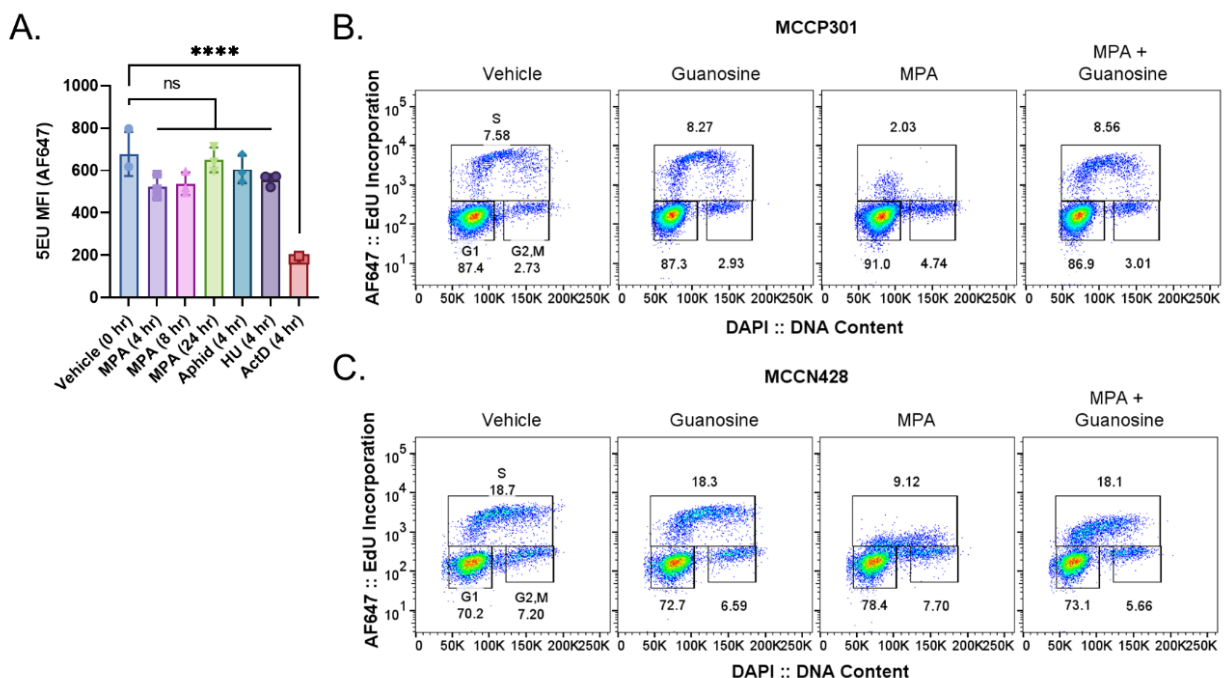


Figure 2.6. Additional nucleotide labeling experiments. (A) Quantification of MFI (AF647) from incorporated 5EU in WaGa single cells treated with MPA (1 μ M), aphidicolin (10 μ M), HU (1 mM) and ActD (1 μ M). N = 3; mean $\pm$ SD; 1-way ordinary ANOVA corrected for multiple comparisons via Tukey *post hoc* test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (B) Flow cytometry diagram of MCCP301 PDCL treated for 4 hours with MPA (5 μ M) and guanosine (1 μ M) and labeled in the final hour with EdU (10 μ M). N = 1. (C) Flow cytometry diagram of MCCN428 PDCL treated for 4 hours with MPA (5 μ M) and guanosine (1 μ M) and labeled in the final hour with EdU (10 μ M). N = 1.

Activation of p53 by IMPDH inhibition depends on the induction of replication stress

The rapid ablation of nascent DNA synthesis following IMPDH inhibition implicated the induction of RS. Indeed, treatment with MPA has been previously reported to induce markers of RS including specific phosphorylation of CHK1, RPA32 and H2AX (Valvezan *et al.*, 2017). However, whether p53 activation upon IMPDH inhibition was dependent on RS was unclear. To determine if IMPDH inhibition induced markers of RS concurrent with p53 activation, we treated WaGa cells for several time points with MPA and monitored protein levels via immunoblot (Figure 2.7A). As early as 4 hours post-treatment, when nascent DNA synthesis was ablated (Figure 2.6C), we observed increased levels of total and phosphorylated p53. In addition, we observed activation of RS markers including phosphorylation of KAP1 (Ser824; pKAP1) (Branigan *et al.*, 2021), ATM-specific CHK2 phosphorylation (Thr68; pCHK2) (Smith *et al.*, 2010), ATR-specific CHK1 phosphorylation (Ser317; pCHK1) (Smith *et al.*, 2010), and DNA-PK-specific RPA32 phosphorylation (Ser8; pRPA32) (Ashley *et al.*, 2014) beginning at 4 to 8 hours with maximal response by 24 hours. The presence of double-stranded DNA breaks (DSBs) as marked by H2AX Ser139 phosphorylation (γ H2AX) was detected at 24 hours. Co-treatment with guanosine reversed accumulation of these RS and DNA damage markers (Figure 2.7B). To determine if p53 activation depended on the stimulation of DNA damage kinases, we treated WaGa cells with MPA in addition to inhibitors of ATR (berzosertib), ATM (KU55933), DNA-PK (AZD7648), or the combination of all three inhibitors (Figure 2.7C). Inhibition of each DNA damage kinase suppressed phosphorylation of their respective downstream targets in response to MPA treatment. Interestingly, ATR inhibition combined with MPA treatment markedly increased accumulation of RS markers relative to MPA alone, an effect not observed with the other kinase inhibitors. In response to individual inhibition of ATR, ATM, or DNA-PK upon MPA treatment, the accumulation and Ser15 phosphorylation of p53 modestly decreased. However, when all three inhibitors were combined, p53 activation was strongly suppressed. These results implicate RS as a major consequence of IMPDH inhibition and further indicate that p53 activation depends on DNA damage kinase activity.

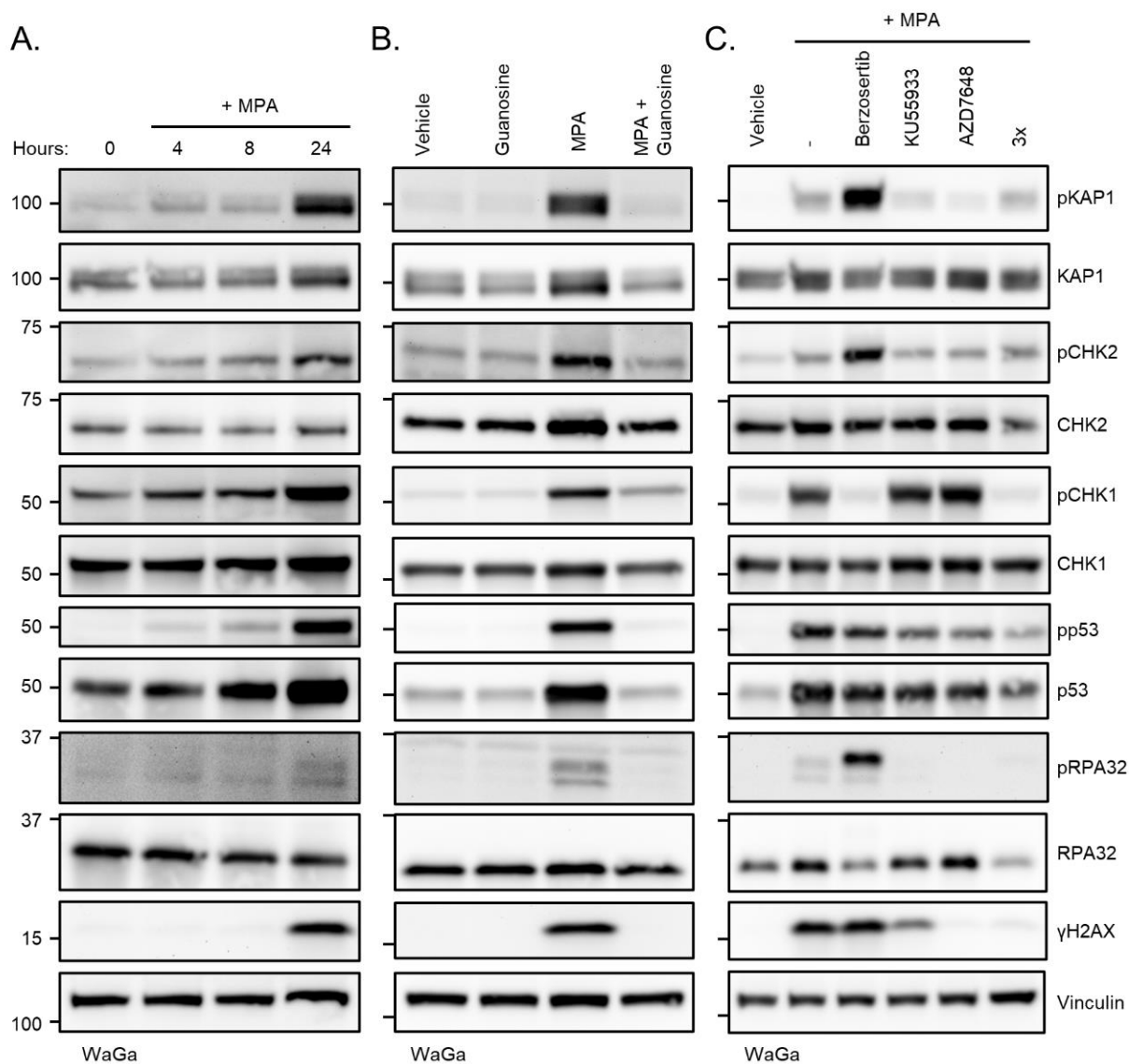


Figure 2.7. Activation of p53 by IMPDH inhibition depends on the induction of RS. (A) Immunoblot of WaGa cells treated up to 24 hours with MPA (1 μ M). Representative of 3 independent experiments. (B) Immunoblot of WaGa cells treated with MPA (1 μ M) and guanosine (10 μ M) for 24 hours. Representative of 3 independent experiments. (C) Immunoblot of WaGa cells treated for 24 hours with MPA (1 μ M) concurrent with inhibitors for ATR (250 nM; berzosertib), ATM (10 μ M; KU55933), DNA-PK (10 μ M; AZD7648) or all 3 (3x). Representative of 3 independent experiments.

Dual-inhibition of IMPDH and ATR induces p53-independent replication catastrophe

Our results implied that suppression of nascent DNA synthesis by IMPDH inhibition led to RS and activation of p53-dependent cytotoxicity. However, MCCN and most other cancers frequently harbor mutant copies of *TP53* (Becker *et al.*, 2017; Kandoth *et al.*, 2013), thereby limiting potential applications. Recent work has shown that dysregulation of the ATR-CHK1 checkpoint in the context of RS can generate extensive DSBs and induce cell death in several cancer models, including those retaining mutant *TP53* (Fokas *et al.*, 2012; Hall *et al.*, 2014; Toledo *et al.*, 2017; Toledo *et al.*, 2013). Since we observed RS as a major outcome of IMPDH inhibition, we hypothesized that combining IMPDH and ATR inhibition could enhance cytotoxicity in cells with non-functional p53.

To test this hypothesis, we induced expression of p53DD or eGFP in WaGa cells, treated these cells with MPA and the ATR inhibitor berzosertib, and then assessed for markers of RS and apoptosis by immunoblot (Figure 2.8A). MPA treatment alone induced accumulation of apoptotic markers that were suppressed by p53DD induction. In this same context, p53DD expression had minimal impact on the accrual of RS markers. Treatment with berzosertib alone suppressed baseline CHK1 phosphorylation (Ser317) but was otherwise unremarkable compared to vehicle. Strikingly, co-treatment with MPA and berzosertib enhanced activation of RS and apoptotic markers excluding pCHK1 over that of MPA alone, an effect independent of functional p53. Furthermore, overexpression of p53DD dramatically increased accumulation of RS markers pKAP1 and pRPA32 relative to eGFP upon dual-treatment. The increased sensitivity to RS in p53-suppressed conditions is consistent with previous reports that demonstrated enhanced sensitivity to ATR inhibitors with the loss of tumor suppressor activity (Barnieh *et al.*, 2021). These effects were similar when assessed by immunoblot in the p53 knockout MKL-1 cells (Figure 2.9A).

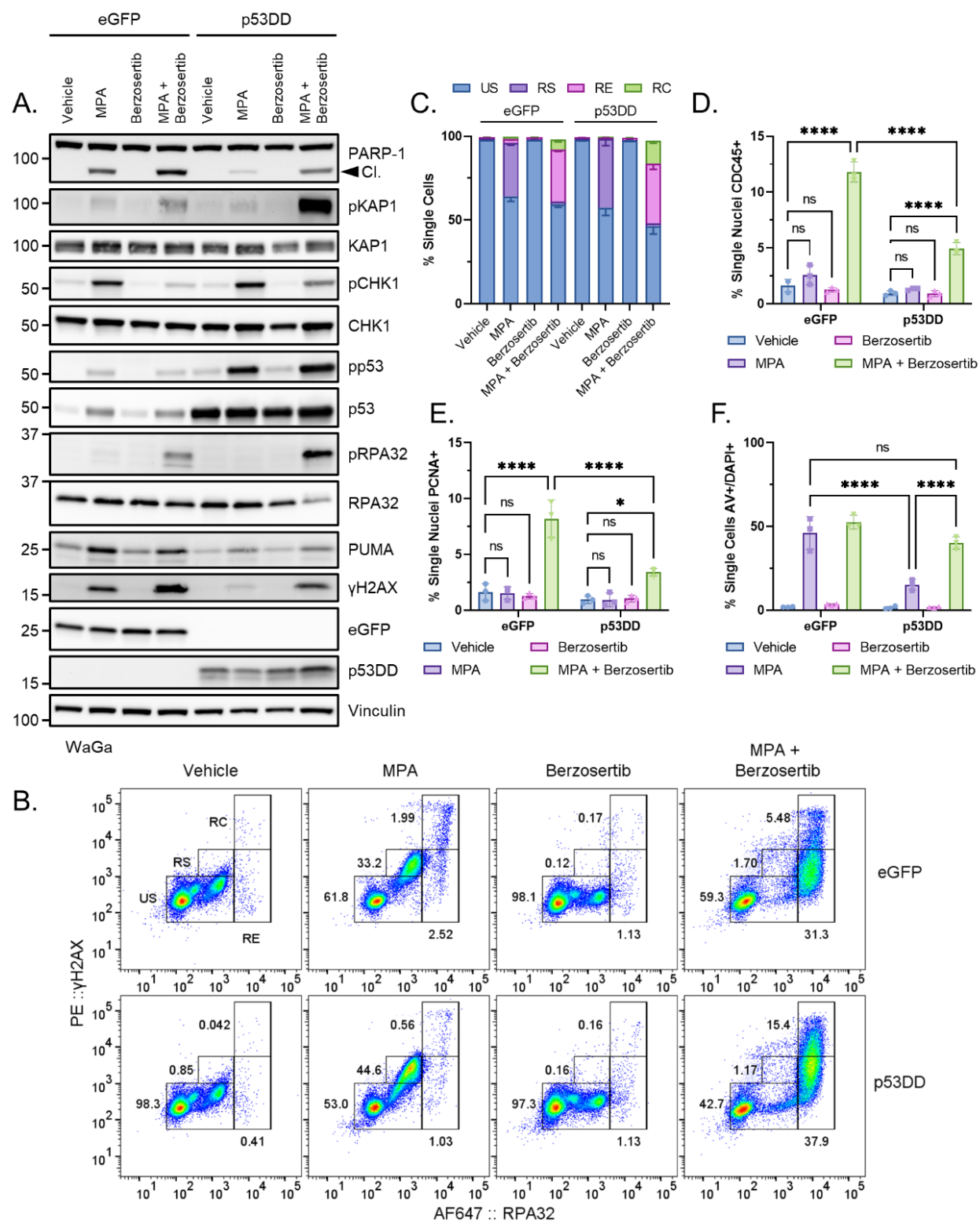


Figure 2.8. Dual inhibition of IMPDH and ATR induces p53-independent replication catastrophe.
 (A) Immunoblot of WaGa cells pre-induced with DOX (1 μ g/mL) for 24 hours to express p53DD or eGFP treated with MPA (1 μ M) and berzosertib (250 nM) for an additional 24 hours. Representative of 3

(Figure 2.8, continued)

independent experiments. (B) Flow cytometry analysis of chromatin-associated γ H2AX and RPA32 in WaGa cells pre-induced and treated as in (A). Gating strategy for each defined population is shown. Images generated in FlowJo. Representative of 3 independent experiments. (C) Quantification of populations from (B). N = 3; mean $\pm$ SD. (D) Quantification of chromatin-associated CDC45 from WaGa cells treated as in (A). N = 3; mean $\pm$ SD; 2-way ordinary ANOVA corrected for multiple comparisons via Tukey *post hoc* test; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. (E) Quantification of chromatin-associated PCNA from WaGa cells treated as in (A). N = 3; mean $\pm$ SD; 2-way ordinary ANOVA corrected for multiple comparisons via Tukey *post hoc* test; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. (F) Quantification of WaGa single cells dual-positive for AV and DAPI prepared as in (A) but treated with inhibitors for 2 days. N = 3; mean $\pm$ SD; 2-way ordinary ANOVA corrected for multiple comparisons via Tukey *post hoc* test; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

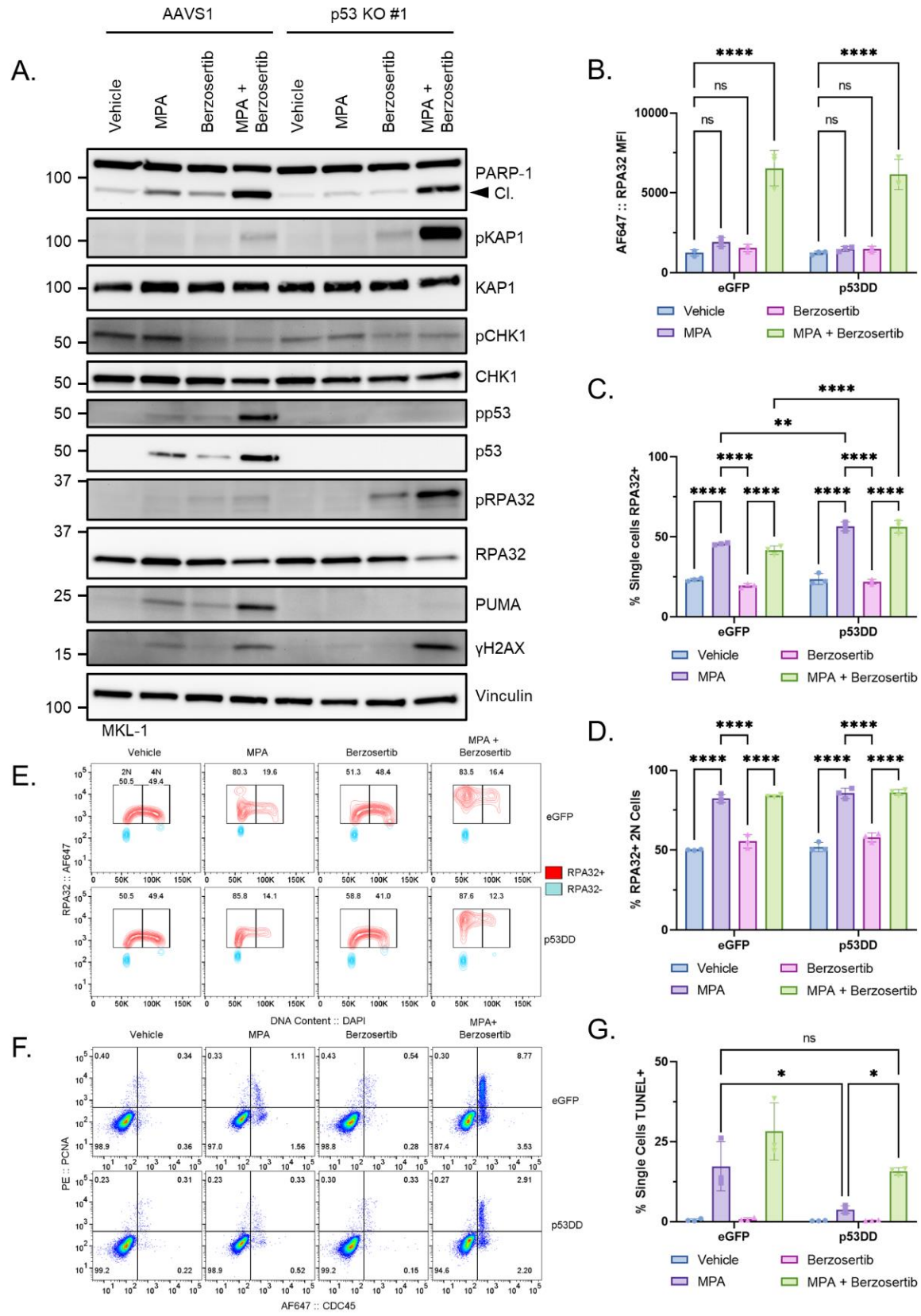


Figure 2.9. Additional replication catastrophe experiments. (A) Immunoblot of p53 knockout MKL-1 cells treated with MPA (1 μ M) and berzosertib (250 nM) for 3 days. Representative of 3 independent experiments. (B) Quantification of MFI (AF647) for chromatin-associated RPA32 in RPA32+ WaGa single cells pre-induced with DOX (1 μ g/mL) for 24 hours to express p53DD or eGFP and treated for an additional 24 hours with MPA (1 μ M) and berzosertib (250 nM). N = 3; mean $\pm$ SD; 2-way ordinary ANOVA corrected for multiple comparisons via Tukey *post hoc* test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (C) Percentage of cells RPA32+ as prepared in (B). Statistical test is identical to (B). (D) Percentage of RPA32+ 2N nuclei as prepared in (B). Statistical test is identical to (B). 2N and 4N populations were gated in eGFP vehicle at a 1:1 ratio (50% 2N / 50% 4N) and applied to each other sample. (E) Flow cytometry contour diagram depicting (D). Representative of 3 independent experiments. (F) Flow cytometry diagram of chromatin-associated CDC45 and PCNA from Figure 5D and 5E. Representative of 3 independent experiments. (G) TUNEL assay of WaGa cells prepared as in (B) but treated for 2 days. N = 3; mean $\pm$ SD; 2-way ordinary ANOVA corrected for multiple comparisons via Tukey *post hoc* test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

To further characterize the mechanism of sensitivity to combined ATR and IMPDH inhibition, we performed chromatin flow cytometry. WaGa cells were induced to express p53DD or eGFP and treated with MPA and berzosertib. Following treatment, cells were pre-extracted with a modified cytoskeletal (CSK) buffer to wash out non-chromatin-associated proteins. The remaining chromatin-associated fraction was fixed, stained, and prepared for flow cytometry. This method allows for the high-throughput, single-cell analysis of a variety of chromatin-bound factors (Branigan *et al.*, 2021; Forment and Jackson, 2015; Toledo *et al.*, 2013).

Fluorescently conjugated antibody staining for chromatin-associated RPA32 and γ H2AX (Toledo *et al.*, 2013) identified cellular populations undergoing RS, RE, or RC upon treatment with MPA and berzosertib (Figure 2.8B, 2.8C). Here we defined RS as the accumulation of DSBs in S-phase cells (γ H2AX+ & RPA32+) in the absence of RE (γ H2AX+/- & RPA32-hi) and RC as the significant accumulation of both marks (γ H2AX-hi & RPA32-hi). Unstressed cells (US) lacked γ H2AX but could be positive for RPA32 (γ H2AX- & RPA32+/-).

MPA treatment alone significantly increased the proportion of cells undergoing RS (~30/40% for eGFP/p53DD, respectively) but did not reach the threshold for RE or RC, regardless of p53 status (Figure 2.8B, 2.8C). In addition, berzosertib treatment did not change cellular populations relative to untreated. However, dual-inhibition of IMPDH and ATR led to a significantly increased proportion of cells undergoing RE (~30/35%, eGFP/p53DD) and RC (~5/15%, eGFP/p53DD) relative to single-treated and untreated cells, irrespective of p53 status. Indeed, the MFI for RPA32 in dual-treated cells was significantly increased, independent of p53 activity (~7-fold; Figure 2.9B). Only RPA32-hi cells were observed in the RC population, in line with previous reports (Toledo *et al.*, 2017; Toledo *et al.*, 2013). We observed that the proportion of S-phase cells (RPA32+) increased upon MPA or dual-treatment (Figure 2.9C). Expression of p53DD significantly increased this population relative to eGFP in paired treatment conditions. Accumulation of RPA32 signal occurred predominantly in cells with diploid (2N) DNA content, despite a block to DNA replication and independent of p53 status (Figure 2.9D, 2.9E). Taken together, these results indicate that combined inhibition of IMPDH and ATR led to significantly increased levels of RPA32- and γ H2AX-bound chromatin resulting in RC that was independent of p53 status.

Inhibition of ATR in the context of RS can lead to increased dormant origin firing, resulting in the massive accumulation of unprotected ssDNA and subsequent induction of RE and RC (Toledo *et al.*, 2013). We sought to further validate this effect by assessing levels of chromatin-associated CDC45 (Figure 2.8D, 2.9F) and PCNA (Figure 2.8E, 2.9F). Chromatin-association of CDC45 and PCNA have been used to assess origin firing and active DNA replication, respectively (Branigan *et al.*, 2021). Single-treatments with MPA or berzosertib did not lead to appreciably increased levels of chromatin-associated CDC45 or PCNA compared to untreated cells. However, dual-treatment induced significant accumulation of both CDC45 and PCNA on chromatin relative to single-treatments or untreated and was independent of p53 status. We observed cells with functional p53 to have increased loading of CDC45 and PCNA relative to those with non-functional p53. This may have been due to a higher proportion of cells with non-functional p53 undergoing RC (15% vs 5%) and thus accruing extensive DNA damage. Activation of DNA replication in the presence of damaged DNA has been shown to induce uncoupling of the replication complex, in part comprised of CDC45 and PCNA, from DNA (Vrtis *et al.*, 2021). Thus, dual-treatment with ATR and IMPDH inhibitors results in dormant origin firing in the presence of a replicative block.

RC is defined as the accumulation of extensive and irreparable DSBs, resulting in cell death (Toledo *et al.*, 2017; Toledo *et al.*, 2013). To determine if dual-treatment could enhance cytotoxicity, we performed AV/DAPI staining (Figure 2.8F) and TUNEL (Figure 2.9G) in p53DD or eGFP-expressing WaGa cells treated with MPA and berzosertib. In both assays, MPA treatment alone significantly increased the proportion of dead cells (AV+/DAPI+ or TUNEL+), an effect that was significantly reduced by p53DD expression. Berzosertib treatment alone did not affect the proportion of dead cells in either assay. Strikingly, p53DD expression did not prevent a significant increase in the proportion of dead cells upon dual-treatment. Indeed, dual-treated p53DD-expressing cells had a similar percentage of dead cells to that of MPA-treated eGFP-expressing cells in both assays. As such, inhibition of ATR sensitizes cells even with non-functional p53 to IMPDH inhibition.

Discussion

Effective treatment options in MCC remain elusive. Although retrospective analysis of clinically advanced MCC showed good response rates to chemotherapy (RR; 55-61%) (Iyer *et al.*, 2016; Tai *et al.*, 2000; Voog *et al.*, 1999), the effect was short-lived with poor progression free survival (PFS; median 94 days) (Iyer *et al.*, 2016). In addition, chemotherapy for MCC can be highly toxic (3.4-7.7% treatment-related deaths) (Tai *et al.*, 2000; Voog *et al.*, 1999), especially since most patients are advanced in age (median 76 years) (Bhatia *et al.*, 2016). As a result, the use of chemotherapy is reserved for palliative conditions. Recent development of anti-PD-L1/PD-1 antibodies for use in metastatic MCC showed promising and durable responses (31.8-56% RR; 40-67% 6-month PFS) with minimal toxicity (Kaufman *et al.*, 2016; Nghiem *et al.*, 2016; Villani *et al.*, 2019). However non-responders and patients refractive to anti-PD-L1/PD-1 therapy, such as those who are immunosuppressed, have minimal recourse (Iyer *et al.*, 2016). As such, novel therapeutic options are needed for these underserved patients.

In this manuscript we demonstrated that IMPDH is a key therapeutic target in MCC. Using a panel of established and patient-derived cell lines encompassing both MCCP and MCCN subtypes, we identified p53 status as a major determinant of sensitivity to IMPDH inhibition. Metabolic assessment by LC-MS and guanosine-based rescue experiments revealed the rapid depletion of GMP nucleotides as the dominant effect of IMPDH inhibition in MCC. We confirmed the requirement of p53 for sensitivity to IMPDH inhibition by functional and genetic perturbations in multiple cell lines. We observed that rapid loss of nascent DNA, but not RNA, synthesis upon IMPDH inhibition correlated with the accumulation of RS and apoptotic cell death. Induction of RS led to DNA damage kinase-dependent p53 activation in response to IMPDH inhibition. Finally, we exploited this RS-inducing effect in combination with ATR inhibition to induce RC and sensitize cells with non-functional p53 to IMPDH inhibition.

Prior work had varied conclusions on whether the dominant effect of IMPDH inhibition was through the suppression of nascent DNA (Valvezan *et al.*, 2017) or RNA synthesis (Huang *et al.*, 2008; Kofuji *et al.*, 2019). Although either nascent DNA or RNA synthesis may be limiting in a specific timeframe, the sequence of inhibition was unclear. Our results indicate that IMPDH inhibition rapidly reduces nascent DNA, but not RNA synthesis. Within hours after IMPDH inhibition we observed the total loss of nascent dNTP incorporation, while nascent NTP incorporation was not significantly affected for up

to 24 hours. It is possible that specific transcripts were reduced. Consistent with prior work (Valvezan *et al.*, 2017), we support the notion that the loss of nascent DNA synthesis is the major effect of IMPDH inhibition.

The chromatin-association assays underscore the utility of flow cytometry for measuring RE and RC. We observed similar chromatin-association kinetics of RPA32 and γ H2AX in conditions expected to induce RE and RC comparable to quantitative image-based cytometry (QIBC) (Toledo *et al.*, 2017; Toledo *et al.*, 2013). In addition, monitoring chromatin-associated RPA32 has proved to be a useful assay to study S phase kinetics for treatments which inhibit DNA synthesis. Previous methods to monitor S phase often function through incorporation of dNTPs and/or staining nuclear content with a DNA-binding dye (Nunez, 2001). These methods, although useful, depend on the incorporation of nucleotides into DNA for measurement. As such, in the context of a block to replication these dynamics can be lost. However, RPA32-coated ssDNA highlights cells currently in S phase independently of dNTP incorporation allowing for a better assessment of replication dynamics.

Identification of p53 as the major effector of IMPDH inhibitor-based sensitivity suggests that stratification of potential responders be based on *TP53* status. Indeed, prior clinical trials involving the use of the IMPDH inhibitor MMF did not assess *TP53* mutation status and, moreover, involved cancers typically containing mutated *TP53* which may have contributed to the lack of reported efficacy (Rodriguez-Pascual *et al.*, 2013; Takebe *et al.*, 2004). A clinical trial with the IMPDH inhibitor tiazofurin in cancers that typically contain wildtype *TP53* (AML & CML-BC) had an encouraging complete response rate (5/16) for end-stage disease that was sustained for 3-10 months following short, intermittent treatment (Tricot *et al.*, 1989). Since MCCP is frequently *TP53* wildtype (Knepper *et al.*, 2019), our work supports further study into the clinical relevance of IMPDH inhibitors in the context of MCCP or, generally, *TP53* wildtype cancers.

Although IMPDH inhibition had *in vitro* efficacy in *TP53* wildtype MCC, MCC with mutant or non-functional p53 had reduced sensitivity. To address this limitation, we exploited the IMPDH inhibition-dependent generation of RS in combination with an ATR inhibitor to enhance cytotoxicity. Utilizing chromatin flow cytometry, we observed the rapid exhaustion of chromatin-bound RPA32 followed by a dramatic increase in chromatin-bound γ H2AX in dual-treated cells that was independent of p53 status.

This induction of RC by co-treatment with an ATR inhibitor re-sensitized MCC with non-functional p53 to IMPDH inhibition. Although treatment with an IMPDH inhibitor alone induced RS irrespective of p53, RPA32 was not exhausted nor was the RC threshold reached. MCC chemotherapy regimens are typically comprised of a topoisomerase II poison combined with an alkylating agent (Iyer *et al.*, 2016; Tai *et al.*, 2000; Voog *et al.*, 1999), which despite being known to inflict RS are unlikely to induce RC as single agents. As such, combining IMPDH or other RS-inducing chemotherapies with ATR inhibition is clinically unassessed in MCC and may improve efficacy. Furthermore, the efficacy of IMPDH and ATR inhibition as a combinatorial therapy warrants further investigation more broadly in cancer.

Methods

Experimental Model and Subject Details

MKL-1 and MS-1 cell lines were a gift from Masahiro Shuda (University of Pittsburgh, PA). The WaGa cell line was a gift from Jürgen Becker (University Duisburg-Essen, Germany). The PeTa cell line was a gift from Rolan Houben (University of Würzburg, Germany). The HEK 293T cells were acquired from the American Type Culture Collection (ATCC). The p53 knockout MKL-1 cell lines were generated by T.C.F., but the manuscript describing their generation is under review elsewhere. The MCCN290, MCCP301, MCCP336, and MCCN350 PDCLs were a gift from Catherine Wu (Dana-Farber Cancer Institute, MA). The MCCN428 cell line was generated de novo from a tumor biopsy as outlined in the methods under the IRB protocol # 09-156. Detailed culturing conditions are detailed in the methods.

Tissue Culture and Cell Line Generation

Established MCC cell lines were cultured in RPMI-1640 supplemented with 10% FBS, 1% PenStrep (Thermo Scientific), and 1% GlutaMAX (Thermo Scientific). HEK 293T cells were cultured in DMEM with 10% FBS, 1% PenStrep, and 1% GlutaMAX. PDCLs were cultured in Neurocult NS-A medium with 10% NS-A supplement, 20 ng/mL FGF (StemCell Technologies), 20 ng/mL EGF (Thermo Scientific), 0.0002% heparin (StemCell Technologies), and 1% PenStrep. All cells were incubated at 37°C in 5% CO₂.

The MCCN428 PDCL was generated in-house from patient-derived tissue. Briefly, patient tumor samples confirmed via histology were washed with PBS and minced into roughly 1 mm pieces before addition into prepared NS-A media (complete) supplemented with 2 mg/mL of both collagenase type-I (Sigma Aldrich) and hyaluronidase type-IV (Sigma Aldrich). Digestion mixtures were incubated for several hours, depending on size and density of sample, with mixing. After most tissue had dissolved, the cellular mixture was run through a 100 µm filter and spun down to isolate mostly single cells. The resulting cells were cultured in NS-A media (complete) and assessed for growth every few days. Isolation of an MCC cell line was confirmed via CK20 immunoblot. Additional PDCLs were generated in a similar manner by the Wu laboratory at the Dana-Farber Cancer Institute.

WaGa cells with inducible eGFP or p53DD were generated as follows. Sequences corresponding to eGFP from pDONR221_eGFP or p53DD from pBabe-hTERT+p53DD were cloned into pLIX-402. Correct insertion was confirmed via Sanger sequencing. Lentivirus was generated in HEK 293T cells via transduction with psPAX2 and pMD2.G. WaGa cells were transduced with the DOX-inducible eGFP or p53DD lentivirus and a polyclonal population was selected with puromycin (1 µg/mL). Inducible expression of eGFP and p53DD was confirmed via immunoblot.

Viability Assays

1000 cells were plated into opaque 96-well tissue culture plates and overlaid with drug to achieve the final concentration listed in a total volume of 100 µL. For the inducible p53DD and eGFP cell lines, cells were pretreated with 1 µg/mL DOX for 24 hours at identical cell concentrations and volumes. The next day, 100 µL of 2x drug concentration were overlaid to generate the final drug concentrations. For the guanosine rescue experiments, drug and guanosine were added simultaneously. In each case established cell lines and PDCLs were incubated for 3 or 5 days, respectively. After incubation, CellTiter-Glo 2.0 reagent was added as recommended (Promega) and luminescence was measured on a M200 Infinite plate reader (Tecan). Alternatively, viable cells were counted on a hemocytometer after addition of equal volumes of cells and 0.4% trypan blue stain.

LC-MS

WaGa cells were plated at 500k per mL into a 12-well dish and incubated for 24 hours before treatment. The next day, drugs were added to the specified final concentrations and returned to the incubator for the listed time. To harvest, cells were washed once in a normal saline solution (0.7 g/L NaCl) and metabolites were extracted via addition of ice-cold (-20°C) 80% MeOH followed by vortexing at 4°C at maximum speed for 10 minutes. Lysates were cleared and the supernatant was dried overnight using a CentriVap (Labconco). The dried metabolites were resuspended in a 1:1 mixture of 20 mM ammonium carbonate and 0.1% ammonium hydroxide (Solvent A) and 100% acetonitrile (Solvent B) and were chromatographically separated by injection into a SeQuant ZIC-pHILIC column (2.1 mm x 150 mm, 5 µm particle size; EMD Millipore) equipped with a guard column (2.1 × 20 mm, 5 µm particle size; EMD

Millipore). The gradient was 0–20 min, linear gradient from 80% to 20% B; 20–20.5 min, linear gradient from 20% to 80% B; 20.5 to 28 min, 80% B. The flow rate was 150 μ L/min. MS full scan was performed in negative ionization mode with m/z range of 70–1000, resolution of 70,000, AGC target 1e6, and maximum integration time of 20 msec. The spray voltage was held at 3.0 kV, heated capillary at 275°C, and HESI probe at 350°C. The sheath gas flow rate was 40 units, the auxiliary gas flow was 15 units, and the sweep gas flow was 1 unit. All LC-MS data were analyzed using EIMaven (v.0.2.4, Elucidata). Compound identification was based on exact mass and retention time matching to commercial standards. Metabolite data were normalized to control conditions.

AV and PI or DAPI cytotoxicity assay

Cells were plated at 500k per mL into 12-well dishes and incubated for 24 hours before treatment. For the inducible p53DD or eGFP cell lines, cells were pretreated with 1 μ g/mL DOX for 24 hours in identical conditions. The next day, drug and/or guanosine were added to the specified final concentrations and returned to the incubator for 48 hours. For the AV/PI stain, the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) was used as per protocol. For the AV/DAPI stain, the PE Apoptosis Detection Kit I (BD Biosciences) was used as recommended except that PI was substituted for 1 μ g/mL DAPI. Cells were filtered through a 70 μ m filter before processing on a LSR Fortessa (BD Biosciences) and analysis via FlowJo.

Immunoblots

Cells were plated at 500k cells per mL into 10 cm dishes and incubated for 24 hours before treatment. The inducible p53DD or eGFP WaGa cell lines were pretreated with 1 μ g/mL DOX for 24 hours in identical conditions. The next day, drug and guanosine were added to the listed final concentrations and returned to the incubator for 24 or 72 hours for the WaGa and MKL-1 cell lines, respectively. After treatment, cells were washed once with PBS and lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (each 1:100, EMD Millipore) in addition to 2- β -mercaptoethanol (1:10,000) for 15 minutes on ice. The resulting lysate was cleared and quantitated via Bradford assay (BioRad). Equal amounts of protein were loaded onto 4-20% polyacrylamide gels and run in a Criterion Cell (BioRad).

Protein was transferred onto a nitrocellulose membrane (BioRad) and blocked in 5% non-fat milk in TBST for 1 hour at room temperature. Primary antibodies were used at the following concentrations overnight in 5% milk in TBST at 4°C with agitation: PARP-1 (1:1000), pKAP1 (1:1000), KAP1 (1:1000), pCHK1 (1:1000), CHK1 (1:1000), pCHK2 (1:1000), CHK2 (1:1000), pp53 (1:1000), p53 (1:1000), pRPA32 (1:1000), RPA32 (1:10 000), PUMA (1:1000), γ H2AX (1:1000), eGFP (1:1000), p53DD (1:500), CASP3 (1:500), β -actin (1:5000), TBP (1:1000), and vinculin (1:30 000). After washing with TBST, anti-mouse or anti-rabbit secondary antibodies conjugated to HRP were incubated at 1:1000 for 1 hour at room temperature in 5% milk in TBST with agitation. After the final incubation, membranes were washed and incubated with either Immobilon (EMD Millipore) or Clarity (BioRad) chemiluminescent substrates and luminescence detected on the G-box system (Syngene). Image analysis was performed in ImageJ.

RT-qPCR

Cells were plated at 500k cells per mL into 6-well dishes and incubated for 24 hours before treatment. The next day, MPA (1 μ M) and guanosine (10 μ M) were added and the plates were returned to the incubator for 24 or 72 hours for WaGa and MKL-1 cells, respectively. After treatment, cells were washed once with PBS and RNA was extracted via TRIzol (Thermo Scientific) as recommended before quantitation with a Nanodrop (Thermo Scientific). 2 μ g of extracted RNA were reversed transcribed via the High-Capacity RNA-to-cDNA Kit (Thermo Scientific) and the resulting cDNA was diluted 1:10 in RNAase-free water. RT-qPCR was performed on the diluted cDNA using the primers listed in the Supplementary Table 1 with the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent) on the Mx Aria (Agilent) system. Relative expression was calculated utilizing the $\Delta\Delta$ Ct method with the geometric mean of β -actin and β -2-microglobulin as controls.

Nucleotide Labeling Flow Cytometry

Cells were plated at 1 million per mL into 12-well dishes and incubated for 24 hours before treatment. The next day, drug and guanosine were added to the specified final concentrations and incubated for the time listed. An hour before harvesting, a final concentration of 10 μ M EdU or 1 mM 5EU was added before returning to the incubator. After treatment, cells were washed once with PBS and then

fixed in 4% MeOH-free formaldehyde in PBS for 15 minutes at room temperature. The fixed cells were washed twice with PBS and permeabilized with ice-cold (-20°C) 70% EtOH overnight. The next day, the permeabilized pellets were washed twice with PBS and incorporated nucleotides were labeled via click-chemistry (0.1 mM THPTA, 100 mM sodium ascorbate, 1 mM CuSO₄, and 1.5 μM AF647 (Click Chemistry Tools)) rotating for 30 minutes at room temperature. After labeling, cells were washed twice with PBS+1% BSA and then stained with 1 μg/mL DAPI in the dark for 40 minutes at room temperature. Cells were washed once more, resuspended in PBS+1% BSA, and filtered through a 70 μm filter before processing on a LSR Fortessa (BD Biosciences). The resulting data was analyzed via FlowJo.

Chromatin Flow Cytometry

Cells were plated at 1 million per mL into 12-well dishes and incubated for 24 hours before treatment. The next day, drugs were added to the specified final concentrations and incubated for 24 hours. After treatment, cells were washed once with PBS before extraction of non-chromatin associated proteins with a freshly prepared, cold (4°C) modified CSK buffer (100 mM sucrose, 100 mM NaCl, 3 mM MgCl₂, 25 mM HEPES (pH 7.3), 0.5% Triton X-100, protease, and phosphatase inhibitors (each 1:100 v/v, EMD Millipore)) for 5 minutes on ice. After extraction, MeOH-free formaldehyde (Thermo Scientific) was added to a final concentration of 4% and incubation was continued on ice for 15 minutes. Permeabilized and fixed cells were washed twice with PBS+1% BSA and incubated overnight in the dark with primary antibodies at the following dilutions: RPA32 (1:250), γH2AX (1:500), CDC45 (1:50), and/or PCNA (1:250). The next day, cells were washed twice with PBS+1% BSA before incubation at room temperature in the dark for 1 hour with either anti-mouse PE or anti-rabbit AF647 conjugate secondary antibodies (each 1:1000). After incubation, a final concentration of 2.5 μg/mL DAPI was added for 40 minutes in the dark at room temperature. Cells were washed one additional time before resuspension in PBS+1% BSA, filtering through a 70 μm filter, and analysis on a LSR Fortessa (BD Biosciences). The resulting data was analyzed via FlowJo.

Quantification and Statistical Analysis

All statistical analysis was performed in GraphPad Prism v9 with recommended settings. All flow cytometry data was analyzed in FlowJo v10.8.1. All LC-MS data was analyzed in EIMaven v.0.2.4. Details for specific statistical tests are available in the figure legends. In this manuscript, N = number of biological replicates performed for a given assay.

Materials Availability

Cell lines or plasmids generated in this manuscript are available upon request.

Data and Code Availability

All data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Chapter 3

Reciprocal expression of YAP1/WWTR1 and neuroendocrine markers in Merkel cell carcinoma

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Figures 3.1, 3.2, 3.3A/B/C, 3.4, 3.5, 3.6, 3.7, 3.9D/E/F/G, 3.10, 3.17B/C, 3.18A/E, 2.19A/D, 2.20C.

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Figures 3.3D, 3.8, 3.9A/B/C, 3.15, 3.16, 3.17A/D/E, 3.18B/C/D, 3.19B/C/E, 3.20A/B.

Mofei Liu: Collected and analyzed patient metadata. Specifically generated the following figures: Table 3.1 & Figures 3.11, 3.12, 3.13, 3.14,

Anita Giobbie-Hurder: Collected and analyzed patient metadata.

Jingwei Cheng: Assisted in the isolation of bulk tumor RNA.

Harita Dharaneeswaran: Collected patient tumor samples and provided patient metadata.

Manisha Thakuria: Collected patient tumor samples and provided patient metadata.

Derin B. Keskin: Provided PDCLs and PDCL RNA-seq dataset.

Catherine J. Wu: Provided PDCLs and PDCL RNA-seq dataset.

James A. DeCaprio: Conceptualized and supervised the study, in addition to editing the manuscript.

*TCF & AKG contributed equally. First-first authorship was decided by coinflip.

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Declaration of Interests

D.B.K owns equity in Agenus, Armata Pharmaceuticals, Breakbio, BioMarin Pharmaceutical, Bristol-Myers Squibb, Celldex Therapeutics, Chinook Therapeutics, Editas Medicine, Exelixis, Gilead

Sciences, IMV, Lexicon Pharmaceuticals, Moderna, and Regeneron Pharmaceuticals. BeiGene, a Chinese biotech company, supports unrelated research at the DFCI Translational Immunogenomics Laboratory (TIGL). C.J.W. holds equity in BioNTech, Inc; and receives research funding from Pharmacyclics. J.A.D. has received research support from Rain Therapeutics. The remaining authors declare no competing interests

Summary

The Hippo pathway transcription factors YAP1 and WWTR1 are frequently oncogenic, and their activation in solid tumors is associated with drug resistance, metastasis, and poor clinical outcomes. In contrast, YAP1/WWTR1 are typically silenced in neuroendocrine (NE) cancers and growth-suppressive when expressed. Merkel cell carcinoma (MCC) is an aggressive NE skin cancer caused by severe UV-induced mutations or expression of Merkel cell polyomavirus (MCPyV) large and small T antigens (LT and ST). Despite genetic differences between the virus-negative and -positive subtypes, clinical diagnostic markers remain identical. As such, the intra-tumoral transcriptional environment resulting in histological homogeneity remains poorly understood. Here we leveraged scRNA-seq of patient-derived tumor biopsies to define a NE transcriptional signature present in both viral subtypes. Assessment of bulk RNA-seq from additional tumor biopsies revealed stratification of MCC upon inversely correlated expression of the NE signature with *YAP1* and *WWTR1*. This inverse correlation was broadly present at the transcript and protein levels in established and patient-derived cell lines. In addition, small populations of YAP1/WWTR1-expressing cells were identified within otherwise NE-hi patient tissues and single cell clusters. Expression of YAP1 or WWTR1 in a NE-hi MCPyV-positive cell line induced cell-cycle arrest through TEAD-dependent transcriptional repression of MCPyV LT. These findings support a model of YAP1/WWTR1 silencing and growth-suppressive activity as a pan-NE cancer phenomenon and further indicate exclusivity between NE- and YAP1/WWTR1-transcriptional programs.

Introduction

Merkel cell carcinoma (MCC) is a neuroendocrine (NE) carcinoma of the skin with two etiological subtypes distinguished by the presence or absence of the integrated Merkel cell polyomavirus (MCPyV) genome (Becker *et al.*, 2017; Feng *et al.*, 2008). Virus-positive MCC (MCCP) minimally expresses the viral large and small T antigens (LT and ST), oncoproteins which are required to transform and sustain the growth of MCCP cells (Houben *et al.*, 2010; Verhaegen *et al.*, 2017). Similar to other polyomaviruses (DeCaprio and Garcea, 2013), MCPyV LT retains an LXCXE motif essential for inhibiting the RB1 tumor suppressor to overcome the G1/S cell-cycle checkpoint (Hesbacher *et al.*, 2016). MCPyV ST induces the formation of a transcriptional co-activator complex to activate expression of genes which contribute to MCC oncogenesis (Cheng *et al.*, 2017), including the p53-targeting E3 ubiquitin ligase MDM2 (Park *et al.*, 2019). Since MCPyV T antigens functionally disrupt the tumor suppressor activity of RB1 and p53, *RB1* and *TP53* are often wildtype in MCCP (Houben *et al.*, 2013; Knepper *et al.*, 2019; Park *et al.*, 2019; Park *et al.*, 2020). By contrast, virus-negative MCC (MCCN) have a highly elevated tumor mutational burden that often includes inactivating mutations in *RB1* and *TP53* (Houben *et al.*, 2013; Knepper *et al.*, 2019; Wong *et al.*, 2015)

Regardless of viral subtype, all MCC have high-grade NE carcinoma histology. Usually, but not always, MCC expresses cytokeratin 20 (KRT20) as well as the NE markers synaptophysin (SYP), chromogranin A (CHGA), and neurofilament (NEFL/M/H) proteins (Bobos *et al.*, 2006; McCardle *et al.*, 2010). Furthermore, MCC expresses numerous markers of normal mechanosensory Merkel cells, including KRT8 and the transcription factors SRY-box transcription factor 2 (SOX2) and atonal BHLH transcription factor 1 (ATOH1) transcription factors (Kervarrec *et al.*, 2019; Moll *et al.*, 1984; Perdigoto *et al.*, 2016) which are essential for survival (Cheng *et al.*, 2017). MCC tumors lacking SOX2 and ATOH1 have not been described, although variable ATOH1 expression levels have been reported (Gambichler *et al.*, 2017) with the presence of MCPyV correlated with increased ATOH1 expression in MCC cell lines (Gravemeyer *et al.*, 2021; Park *et al.*, 2020). Nonetheless, a complete picture of how viral-status and NE gene expression may interact to define MCC subtypes remains to be described.

The Hippo pathway is a signaling cascade comprised of the mammalian STE20-like protein (MST1/2) and large tumor suppressor (LATS1/2) kinases which directly regulate the Yes1-associated

transcriptional regulator (YAP1) and WW domain-containing transcription regulator 1 (WWTR1) transcription factors (Piccolo et al., 2014). In response to growth-suppressive conditions the MST1/2 and LATS1/2 kinases become sequentially activated, resulting in LATS1/2-dependent phosphorylation of YAP1 or WWTR1 thereby marking them for poly-ubiquitination and degradation or retention in the cytosol (Koo and Guan, 2018). Under growth-promoting conditions the MST1/2 and LATS1/2 kinases are inactive, thereby allowing YAP1 and WWTR1 to enter the nucleus and complex with DNA-binding transcription factors such as TEA domain transcription factors (TEAD1-4) (Koo and Guan, 2018). YAP1/TEAD and WWTR1/TEAD complexes have been described as promoting both G1/S and G2/M gene expression (Pattschull et al., 2019; Zanconato et al., 2015), but have additional activities and targets depending on their specific binding partners.

Aberrant activation of YAP1/WWTR1 has been reported for several solid tumors including breast, gastric, and non-small cell lung cancer. In these cancers, YAP1/WWTR1 expression is associated with increased drug resistance, metastasis, and poor outcomes (Yu et al., 2018; Zanconato et al., 2016). By contrast, transcript-level silencing of *YAP1/WWTR1* has been reported in hematological cancers such as multiple myeloma (MM) (Cottini et al., 2014; Grieve et al., 2019) and NE cancers such as medullary thyroid cancer (Celano et al., 2018; Galuppini et al., 2017; Hudson et al., 2013), NE prostate cancer (Cheng et al., 2020), and small cell lung cancer (SCLC) (Horie et al., 2016; Ito et al., 2016; McColl et al., 2017; Nishikawa et al., 2011). Expression of YAP1 or WWTR1 in MM and SCLC cell lines in which *YAP1/WWTR1* are silenced at baseline leads to decreased cell viability (Cottini et al., 2014; Grieve et al., 2019; Nishikawa et al., 2011). Nonetheless a variant SCLC subtype has been described that expresses YAP1, albeit with reciprocal lower expression of the NE transcription factor achaete-scute family BHLH transcription factor 1 (ASCL1) (Horie et al., 2016; Ito et al., 2016; McColl et al., 2017). Furthermore, SCLC exhibits intra-tumoral heterogeneity where ASCL1-hi or SYP-hi tumors contain small subpopulations of YAP1-expressing cells, suggesting transcriptional plasticity in the NE transcriptional program (Ireland et al., 2020; Pearsall et al., 2020). Another recent study suggested *YAP1* silencing and YAP1-dependent growth-suppressive activity is a pan-NE cancer phenomenon (Pearson et al., 2021), but this remains poorly studied in NE cancers other than SCLC.

Here we show that MCC can be distinguished by inversely correlated expression of YAP1/WWTR1 and NE-associated genes. Moreover we find that MCC, like SCLC, exhibits intra-tumoral heterogeneity in which NE-hi tumors contain a subpopulation of YAP1/WWTR1-expressing cells. Finally, we show that expression of YAP1 or WWTR1 in a NE-hi MCCP cell line decreases cell viability and enforces cell-cycle arrest through TEAD-dependent transcriptional suppression of MCPyV LT. Our work supports the notion that *YAP1/WWTR1*-silencing and growth-suppressive activity is a pan-NE cancer phenomenon and further supports the exclusivity between NE- and YAP1/WWTR1-transcriptional programs.

Results

Single-cell and bulk RNA-seq analysis of MCC tumor biopsies reveals NE-hi and -lo subtypes

To assess the transcriptional environment present within MCC tumors, we performed single-cell RNA-seq (scRNA-seq) on 9 patient-derived tumor biopsies to obtain 38,077 high quality cells (Figure 3.1A). Of the 9 tumor samples, 6 were virus-positive with 25,062 cells defined by non-zero expression of MCPyV *LT* or *ST*, and 3 were virus-negative with 13,015 cells (Figure 3.2A). All samples were integrated into a single clustering analysis using Seurat (Hao et al., 2021) to identify pan-MCC cellular subgroupings (Figure 3.1A). Seventeen unique transcriptional clusters were identified (resolution = 0.75), the majority of which (clusters 0-6, 8-10, 12-15, representing 89.4% of all cells) were broadly categorized as MCC as defined by general expression *ATOH1*, *SOX2*, *KRT20* and common NE-markers including *SYP*, *CHGA*, and *NEFH* (Figure 3.1B). In addition to MCC cells, we also observed clusters of immune cells including CD4⁺ and CD8A⁺ T cells (clusters 7 and 11) in addition to CD68⁺ macrophages (cluster 16). Each of these clusters were broadly present within each sample, though relative proportions varied (Figure 3.2B). Importantly, the heterogeneity of MCC cell clustering was not due to viral-status as we observed dispersed co-clustering of MCC cells from virus-positive and -negative samples (Figure 3.1C). Thus, we wondered if factors other than viral-status was driving transcriptional heterogeneity in MCC.

To assess transcriptional drivers of the different MCC clusters, we identified genes significantly enriched within a single cluster relative to all other clusters (Supplemental Table 1). We further assessed these cluster-enriched genes for conservation across all tumor specimens to identify high-confidence, cluster-specific enrichment (Supplemental Table 2). Interestingly, cluster 0 was enriched for the expression of six genes relative to each other cluster and whose enrichment was also conserved across all tumor samples. These genes included markers of normal Merkel cells including *ATOH1*, *SOX2*, and *KRT8*; HES family BHLH transcription factor 6 (*HES6*) and ISL LIM Homeobox 1 (*ISL1*), previously shown to be highly expressed in MCC and other NE cancers (Agaimy et al., 2013; Gravemeyer et al., 2021; Harold et al., 2019); and coiled-coil glutamate rich protein 2 (*CCER2*), previously not associated with MCC or NE transcriptional programs. As a result, we reasoned that cluster 0 represented the prototypic NE MCC cell population. Importantly, cluster 0-enriched genes were broadly, though variably, expressed

in MCC but not immune cell clusters (Figure 3.1D). These results indicated that the transcriptional heterogeneity within MCC may be based upon NE gene expression, rather than viral-status.

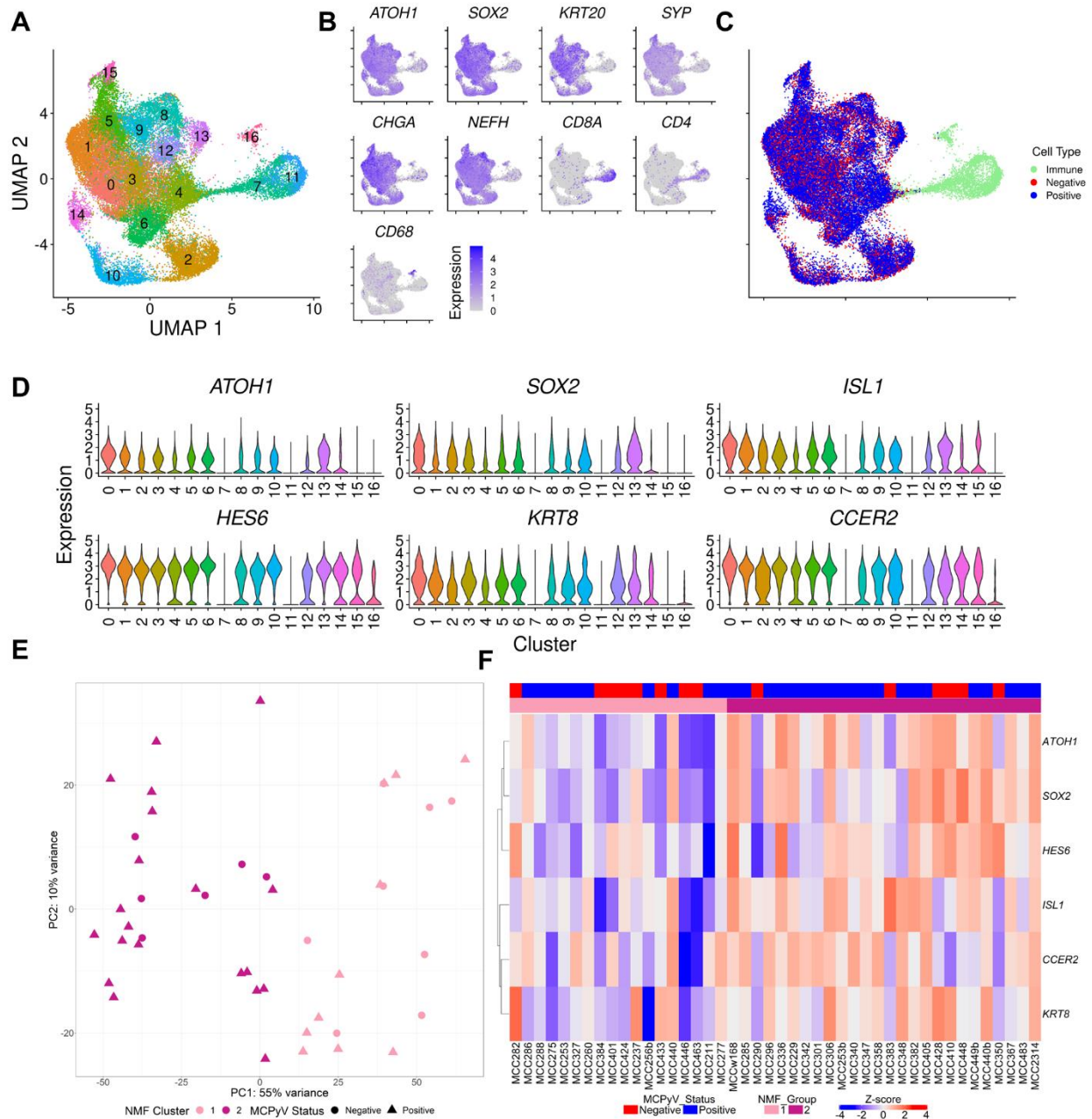


Figure 3.1. Single-cell and bulk RNA-seq of patient-derived MCC tumor biopsies identifies novel subgroups based on expression of NE genes. A. Dimensional reduction plot (DimPlot) of integrated scRNA-seq analysis of all cells from 9 patient-derived tumor biopsies with clusters defined (resolution = 0.75). B. Feature plots of MCC (*ATOH1*, *SOX2*, *KRT20*), NE (*SYP*, *CHGA*, *NEFH*), and immune cell markers (*CD8A*, *CD4*, *CD68*). C. DimPlot as in (a) but colored based upon MCPyV-status of the tumor biopsy or presence in the immune cell clusters. D. Violin plots comparing normalized expression of conserved cluster 0 genes between clusters. E. PCA of bulk RNA-seq from 44 patient-derived MCC tumor biopsies showing viral status (shape) and NMF group (color). F. Expression of each cluster 0 gene in the 44 MCC tumor biopsies clustered by NMF group.

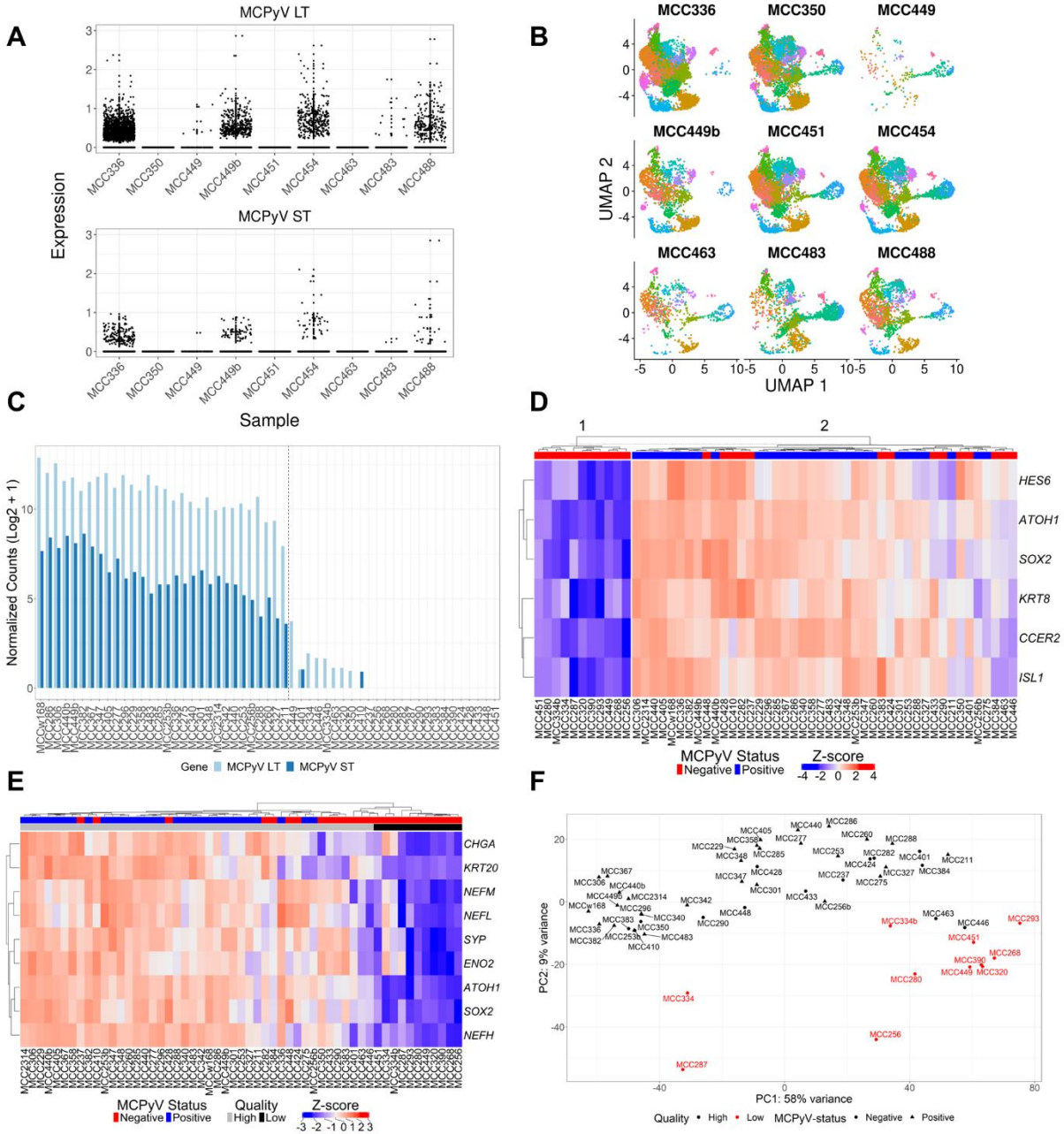


Figure 3.2. Viral status determination and quality control for scRNA-seq and bulk tumor biopsy RNA-seq. A. MCPyV *LT* and *ST* normalized expression in each tumor sample assayed by scRNA-seq. Samples with nonzero expression of both *LT* and *ST* were deemed virus-positive. B. DimPlot of cells representing clusters present within each of 9 tumor samples assayed by scRNA-seq. C. Log2 + 1 normalized counts of MCPyV *LT* and *ST* transcripts in each of the 55 tumor samples analyzed by bulk RNA-seq. Cutoff of 100 normalized *LT* counts and 10 normalized *ST* counts were used to call tumors virus-positive or virus-negative. Line denotes samples meeting the cutoff. D. Heatmaps for expression of cluster 0 and E. NE/MCC genes in the 55 bulk RNA-sequenced tumor samples. The 11 samples which lacked robust expression of the cluster 0 and canonical MCC genes (black) were removed from further analysis. F. PCA of all 55 bulk RNA-sequenced tumor samples highlighting the 11 samples removed (red).

To further probe transcriptional heterogeneity in MCC, we performed bulk transcriptional profiling on 55 patient-derived tumor biopsies, including paired tissue samples for seven of the nine samples analyzed by scRNA-seq (Supplemental Table 3). Viral-status was determined by the expression of MCPyV *LT* and *ST* (Figure 3.2C). Since tumor biopsies can contain normal skin or immune cells, we curated our dataset for higher purity tumor samples based on the expression of the cluster 0-enriched genes defined in the scRNA-seq (Figure 3.2D). Eleven of the 55 samples had minimal expression of these as well as other NE genes and MCC-specific markers (Figure 3.2E). Both Euclidean clustering (Figure 3.2D, 3.2E) and principal component analysis (PCA; Figure 3.2F) showed divergence of these samples from the remaining 44, suggesting a large proportion of non-tumor cells. These samples were removed from further analysis. PCA of the top 500 most variable genes (Supplemental Table 4) was used to assess the clustering of the 44 remaining samples (Figure 3.1E). Similar to the scRNA-seq, we observed co-clustering of virus-positive (triangles) and-negative (circles) tumors samples. To identify transcriptional subgroupings within the bulk RNA-seq dataset, we performed non-negative matrix factorization (NMF) of the top 500 most variably expressed genes. We imputed a range of factorization ranks (k) to define the ideal value for subgroup decomposition (Brunet et al., 2004), which was optimized at $k = 2$ (cophenetic correlation = 0.952) indicating two groups. Importantly, these NMF-based transcriptional groups did not correlate with viral-status (Figure 3.1E).

Since we observed variability in NE gene expression in the scRNA-seq, we wondered if the NMF-defined groups may diverge based upon expression of the NE cluster 0-enriched genes (Figure 3.1F). Generally, NMF group 1 (pink) was associated with lower expression of cluster 0 genes while group 2 (purple) was associated with higher expression. However, a gradient of cluster 0 gene expression was apparent, suggesting that groups 1 and 2 likely represent two overlapping transcriptional profiles or a spectrum of NE gene expression.

YAP1/WWTR1 and NE marker expression are negatively correlated in MCC on a bulk and single-cell basis

Since analysis of the scRNA-seq and bulk RNA-seq datasets revealed heterogeneity of NE gene expression within and across tumors, we reasoned that genes whose expression was negatively correlated with the cluster 0 genes may help to define the NMF group 1 tumor samples. Genome-wide, gene-to-gene Pearson correlations were performed on the bulk RNA-seq dataset using the six cluster 0 genes (Figure 3.3A; Figure 3.4A; Supplementary Table 5). Among the most negatively correlated genes compared to *SOX2* and *ATOH1* were *YAP1* (R^2 : -0.62, -0.66, respectively) and *WWTR1* (R^2 : -0.59, -0.61, respectively). *YAP1* and *WWTR1* expression was also negatively correlated with expression of the other cluster 0 genes (Figure 3.4A). Intriguingly, expression of *YAP1* has been used to define a rare, non-NE subtype in the related NE cancer, SCLC (Horie *et al.*, 2016; Ito *et al.*, 2016; McColl *et al.*, 2017; Pearsall *et al.*, 2020). Additionally, prior IHC analysis showed lack of *YAP1* expression in MCC and other NE carcinomas with high expression of NE markers (Muto *et al.*, 2021). As such, we postulated that some of the heterogeneity observed in the scRNA-seq and bulk RNA-seq could be explained by the reciprocal expression of the NE cluster 0 genes relative to non-NE-associated *YAP1* and *WWTR1*.

Reassessment of the scRNA-seq dataset revealed a small population of cells in clusters 8 and 12 that expressed *YAP1*, *WWTR1*, and their direct transcriptional targets cellular communication network factor 1 (*CCN1* or *CYR61*) and cellular communication network factor 2 (*CCN2* or *CTGF*; Figure 3.4B). Indeed, *WWTR1* was significantly enriched in cluster 12 relative to all other clusters (Supplemental Table 1). These *YAP1/WWTR1*-expressing cells were rare but present in all tumor specimens, albeit at different proportions (Figure 3.4C, 3.4D) and were not restricted to NMF or viral subtypes. In general, *WWTR1* was more strongly expressed than *YAP1* and expressed by more cells per sample across all samples (Figure 3.4D). Expressional analysis of all *WWTR1*-positive single-cells revealed a clear negative correlation between the NE cluster 0- and *YAP1/WWTR1*-associated gene sets (Figure 3.3B). Importantly, we observed low but present expression of *WWTR1* in single cells with a high expression of the cluster 0 markers (Figure 3.3B, left), indicating that *WWTR1* expression was present in NE MCC cells albeit at reciprocally lower levels. Furthermore, we also found *KRT8*, a marker of normal Merkel cells and MCC, co-expressed in single cells with higher expression of *WWTR1* (Figure 3.3B, center) indicating that individual MCC cells can express *WWTR1*. Taken together, these results indicate that *YAP1/WWTR1*

expression can be observed in *bona fide* MCC at the single-cell level and broadly negatively correlate with NE gene expression.

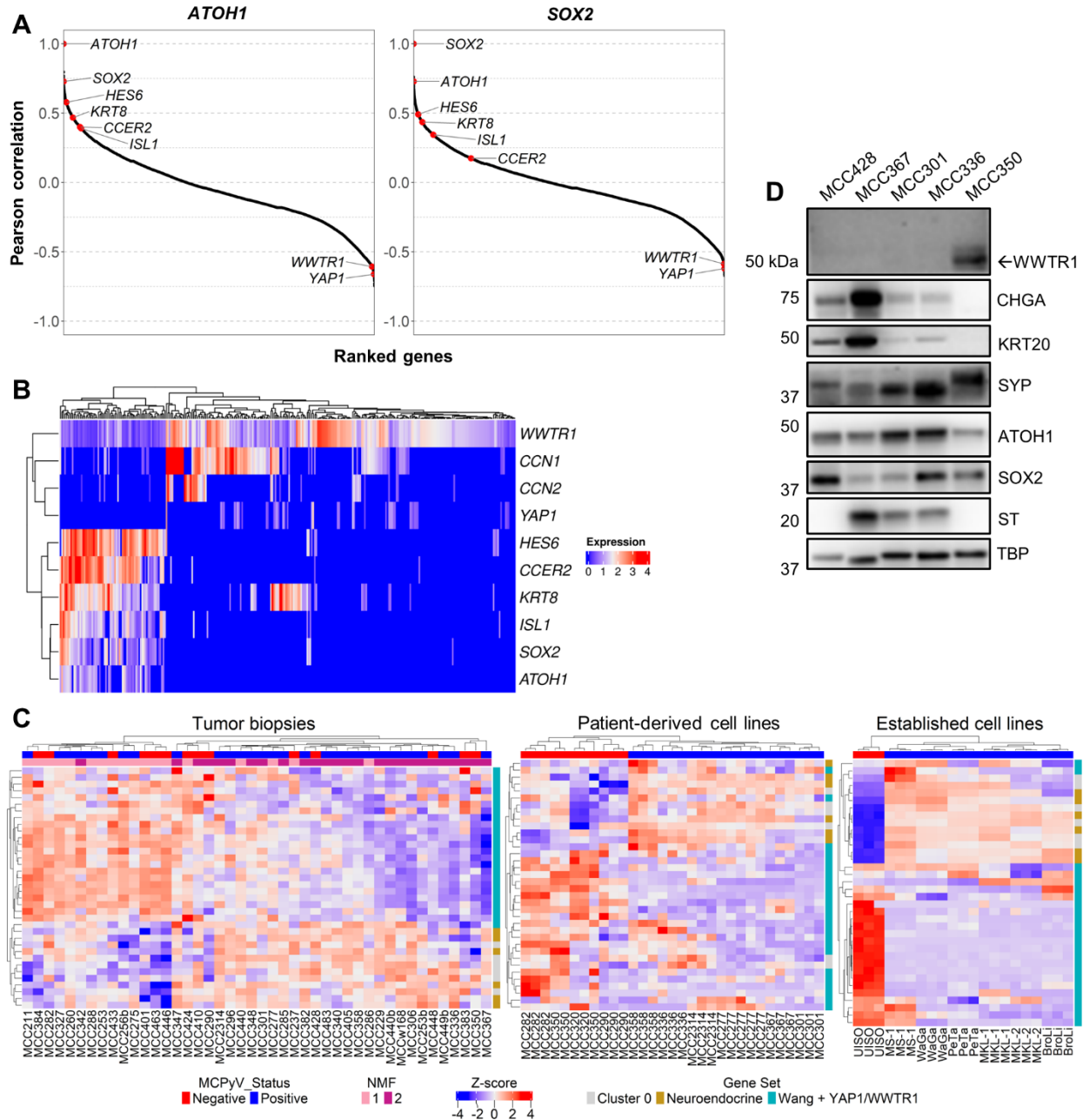


Figure 3.3. NE marker and YAP1/WWTR1 expression are strongly negatively correlated in MCC tumor samples. A. Genome-wide gene-to-gene Pearson correlations for 44 bulk RNA-sequenced tumor samples showing *YAP1* and *WWTR1* are negatively correlated with *ATOH1* and *SOX2* across all samples. B. Heatmap of normalized expression of *YAP1*, *WWTR1*, *CCN1*, *CCN2*, and cluster 0 genes in each *WWTR1*-expressing cell (N = 301) from the 9 tumor samples analyzed by scRNA-seq. C. Heatmaps of the 44 bulk RNA-sequenced tumor samples (left), RNA-sequenced PDCLs (middle), and bulk RNA-sequenced DMSO-treated established MCC cell lines (right) showing negatively correlated expression between *YAP1/WWTR1* and their target genes, cluster 0 genes, and other NE genes. D. Immunoblot of PDCLs showing negatively correlated expression between *WWTR1* and NE markers. N = 1.

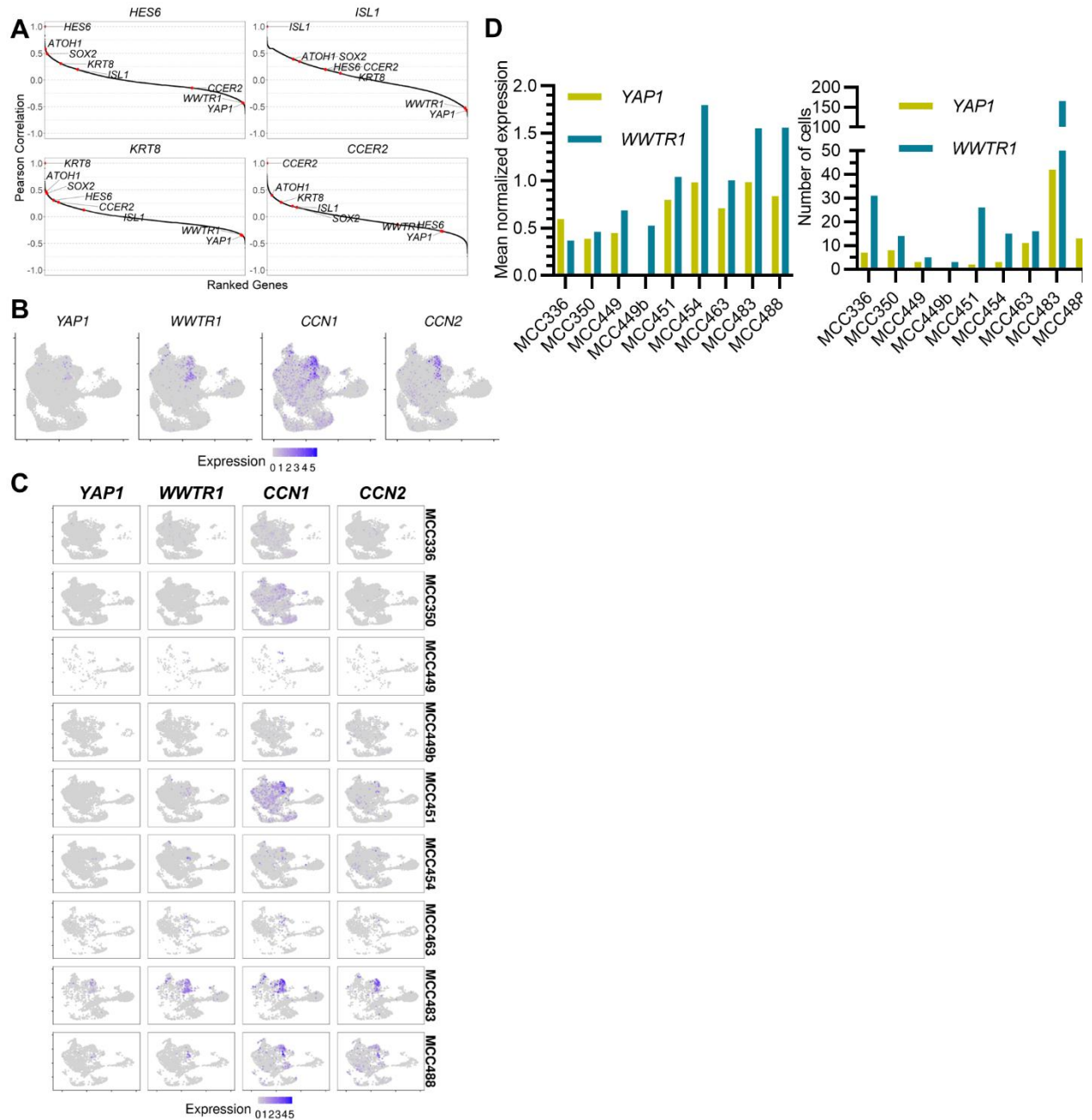


Figure 3.4. Analyses of YAP1/WWTR1 expression in patient-derived MCC tumor biopsies. A. Genome-wide gene-to-gene Pearson correlations for 44 bulk RNA-sequenced tumor samples highlighting correlations of *HES6*, *ISL1*, *KRT8*, and *CCER2* with *YAP1* and *WWTR1* across all samples. B. Feature plots showing expression of *YAP1*, *WWTR1*, and their targets *CCN1* and *CCN2* in the scRNA-seq. C. Feature plots of *YAP1*, *WWTR1*, *CCN1*, and *CCN2* in each of the 9 tumor samples assessed by scRNA-seq. D. Number of *YAP1*- and *WWTR1*-expressing cells and mean normalized expression values for positive cells across all samples.

We asked whether the reciprocal expression of *YAP1/WWTR1* and NE markers was observed in the bulk RNA-seq of the 44 tumor samples. We plotted expression of the NE cluster 0 genes, *KRT20*, other NE markers including *CHGA*, *SYP*, and *NEFL/M/H*, as well as *YAP1*, *WWTR1*, and a curated list of 21 known direct transcriptional targets of *YAP1/WWTR1* ("Wang targets"; Figure 3.3C) (Wang et al., 2018). Not only was the negative correlation between the cluster 0/NE markers and *YAP1/WWTR1*-associated genes clearly observed, but there was also good agreement with the NMF grouping. *YAP1/WWTR1* and their targets were more highly expressed in group 1 tumors, allowing us to define this group as NE-lo and group 2 as NE-hi.

IHC was performed on a panel of MCC tumors to determine whether subpopulations of *YAP1/WWTR1*-expressing cells could be observed *in situ*. In line with previous reports (Gravemeyer et al., 2021; McCardle et al., 2010), all H&E-positive tumor sections stained for ATOH1 (7/7) and most for KRT20 (5/7; Figure 3.5, 3.6A). However, expression of ATOH1 and KRT20 varied in intensity and positivity within individual tumor sections, indicating the potential for heterogenous subpopulations (Figure 3.6B, 3.6C). Indeed, staining for *WWTR1* was rare but present in several samples (3/7, MCC384 used as example) within otherwise H&E- and ATOH1-positive tumor sections (Figure 3.7, red arrows). *YAP1*-positive cells were exceedingly rare but present in at least one sample (MCC384) within the *WWTR1*-positive region (Figure 3.7, red arrows). Importantly, MCC384 contained nuclear staining of *WWTR1* and *YAP1*, suggesting the potential for *WWTR1/YAP1*-mediated transcriptional activity.

In addition to the 44 tumor samples, reciprocal expression of the *YAP1/WWTR1*-associated and cluster 0/NE gene sets was observed in transcriptional profiling of patient-derived cell lines (PDCLs; Figure 3.3C; Supplemental Table 6). There was general concordance between a PDCL's transcriptional pattern and its associated tumor's NMF grouping, with 5/6 NE-hi PDCLs having associated group 2 tumor samples and 1/3 NE-lo PDCLs having an associated group 1 tumor sample. Two of the PDCLs which differed from their associated tumor sample in classification were MCC290 and MCC350, which clustered with the NE-lo cell lines despite having associated group 2 tumor samples. Differences in expression pattern may reflect cell lines derived from sub-populations of *YAP1/WWTR1*-expressing cells within the tumor or from culture conditions.

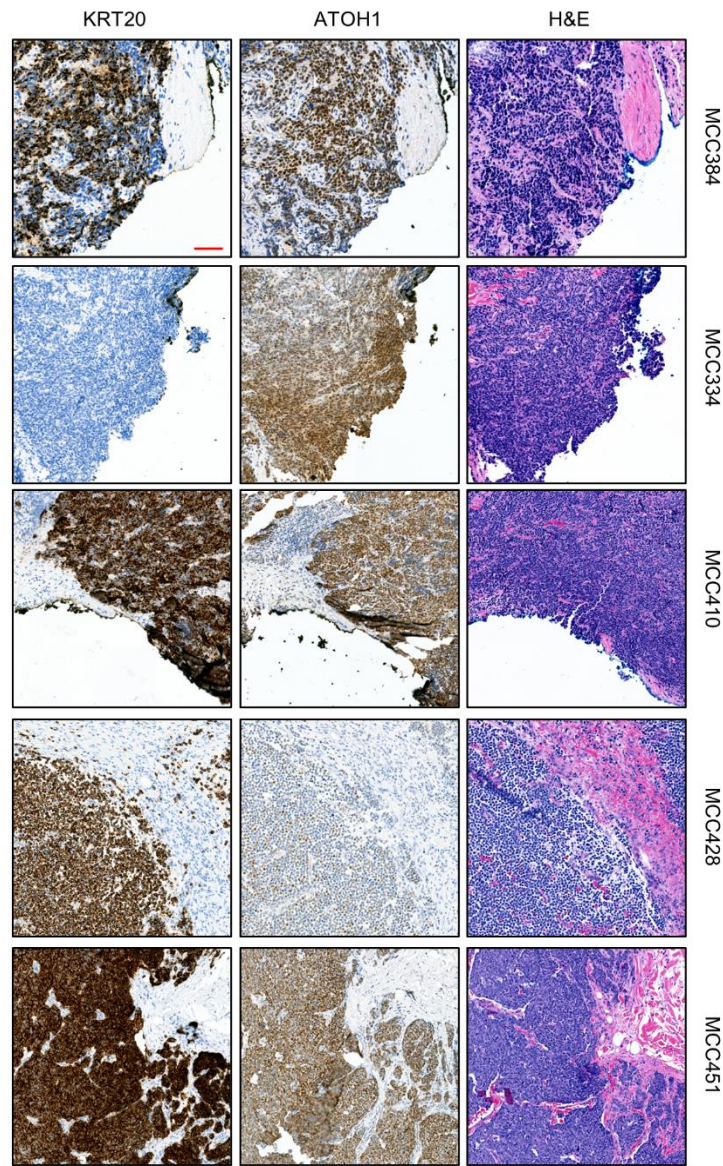


Figure 3.5. IHC of patient-derived tumor biopsies for H&E, ATOH1, and KRT20 markers. Images were captured at 20x magnification. Red scale bar represents 100 μ m.

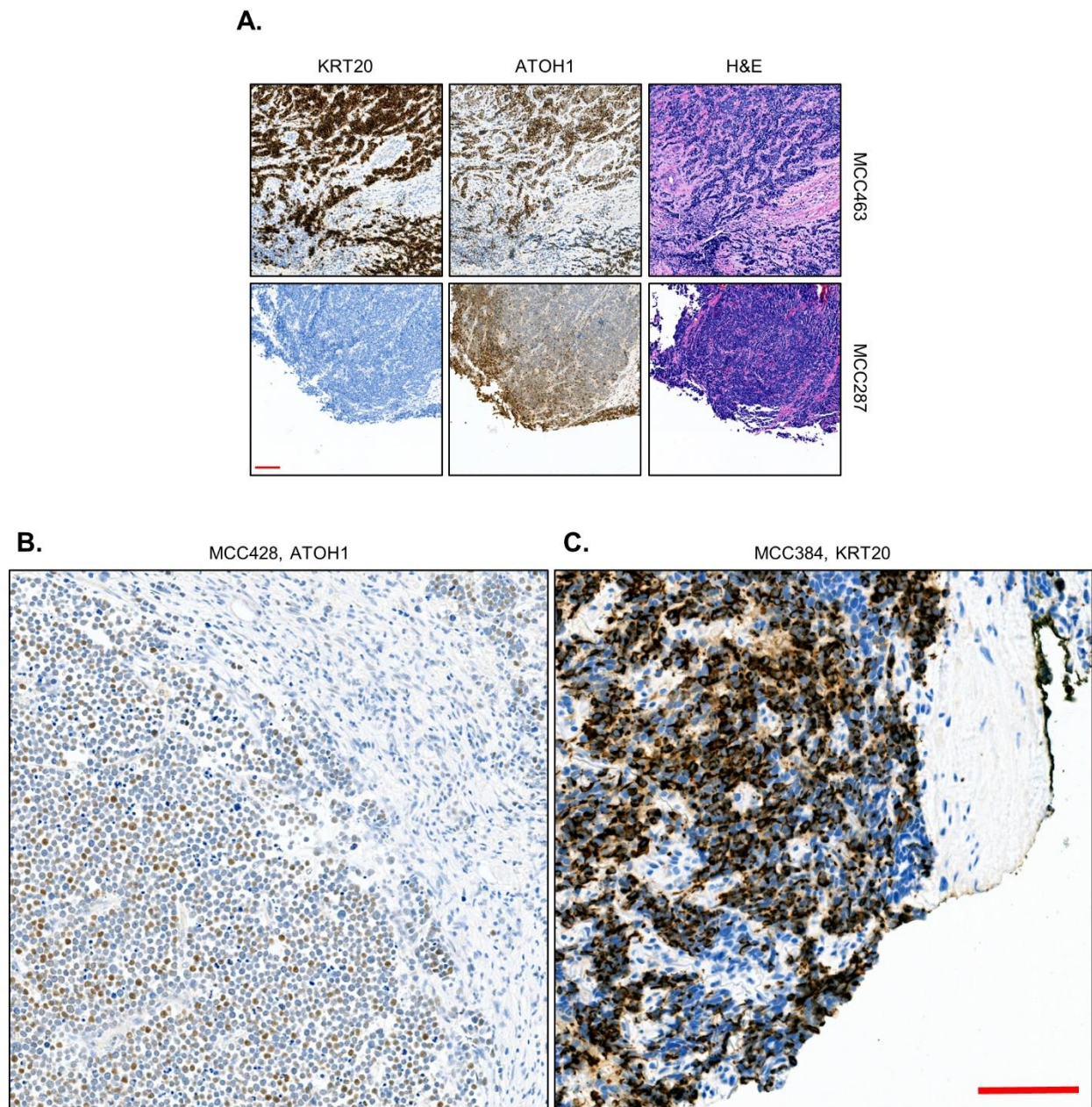


Figure 3.6. Additional IHC analysis of patient-derived tumor biopsies. A. Images processed and analyzed as in Figure 3.5. B. Enlarged image of representative tumor section in MCC428 for ATOH1 staining. 40x magnification; identical to Figure 3.5. C. Enlarged image of representative tumor section of MCC384 for KRT20 staining. 40x magnification; identical to Figure 3.5. Red scale bar represents 100 μm .

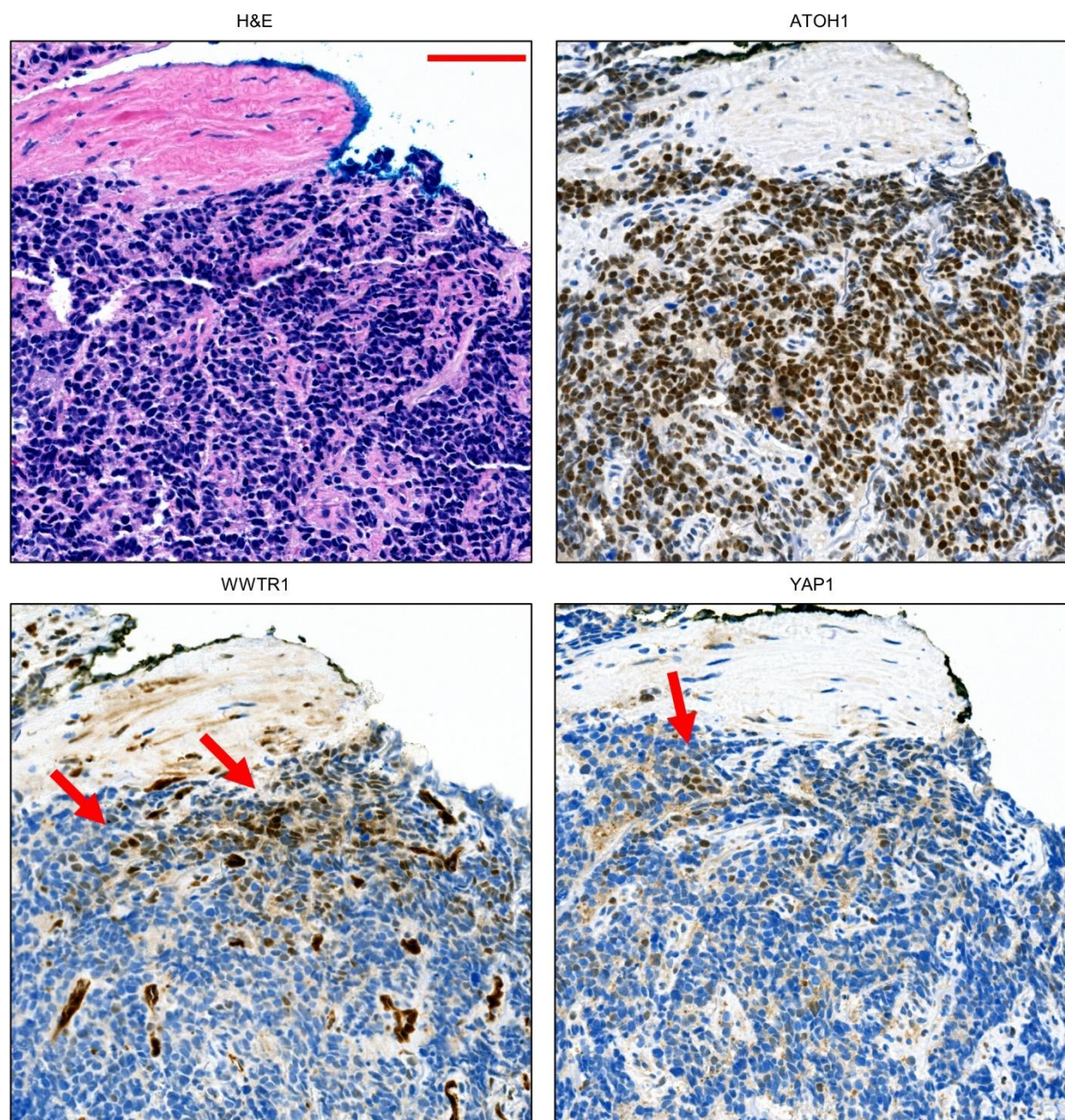


Figure 3.7. IHC of WWTR1- and YAP1-positive patient-derived tumor biopsy section. Images are from MCC384 including H&E (top left), ATOH1 (top right), WWTR1 (bottom left), and YAP1 (bottom right). Magnification 40x. Red scale bar represents 100 μ m. Red arrows denote WWTR1- or YAP1-positive cells.

RT-qPCR analysis of *YAP1/WWTR1* in three PDCLs confirmed the RNA-seq findings (Figure 3.8A). Immunoblot analysis showed expression of WWTR1 in the MCCN PDCLs MCC350 (Figure 3.3D) and MCC410 (Figure 3.8B). Notably, the MCC350 and MCC410 PDCLs had reciprocally lower expression of NE markers including CHGA and ATOH1 compared to the other PDCLs. YAP1 was not detectable in any of the PDCLs. We noted that the PDCL RNA-seq displayed a stronger relationship between transcriptional status and viral-status than the tumor sample RNA-seq, with only MCCN lines being classified as NE-lo. However, not all MCCN cell lines expressed WWTR1 protein, as exemplified by MCC428 (Figure 3.3D).

We also examined previously published RNA-seq datasets (Park *et al.*, 2020) of the established MCC cell lines MKL-1, MKL-2, MS-1, WaGa, PeTa, BroLi, and UISO treated with DMSO (Figure 3.3C). The negative correlation between expression of YAP1/WWTR1-associated genes and cluster 0/NE genes was also observed in this dataset. We confirmed these findings for a subset of the cell lines, in addition to the virus-negative lines MCC13 and MCC26, via RT-qPCR (Figure 3.8C) and immunoblot (Figure 3.8D). In contrast to the PDCLs, YAP1 protein was observed in the three WWTR1-positive, established cell lines. We noticed that the reciprocal expression pattern between YAP1/WWTR1 and the cluster 0/NE genes as well as correlation with viral-status was more evident in the established cell line RNA-seq than it was for the PDCLs or 44 tumor samples. In addition, the core Hippo pathway kinases were expressed in all of the established cell lines (Figure 3.8E) and PDCLs (Figure 3.8B), including ones that did not express YAP1 or WWTR1, and most cell lines had some degree of MOB1 phosphorylation, an indicator of Hippo pathway activation.

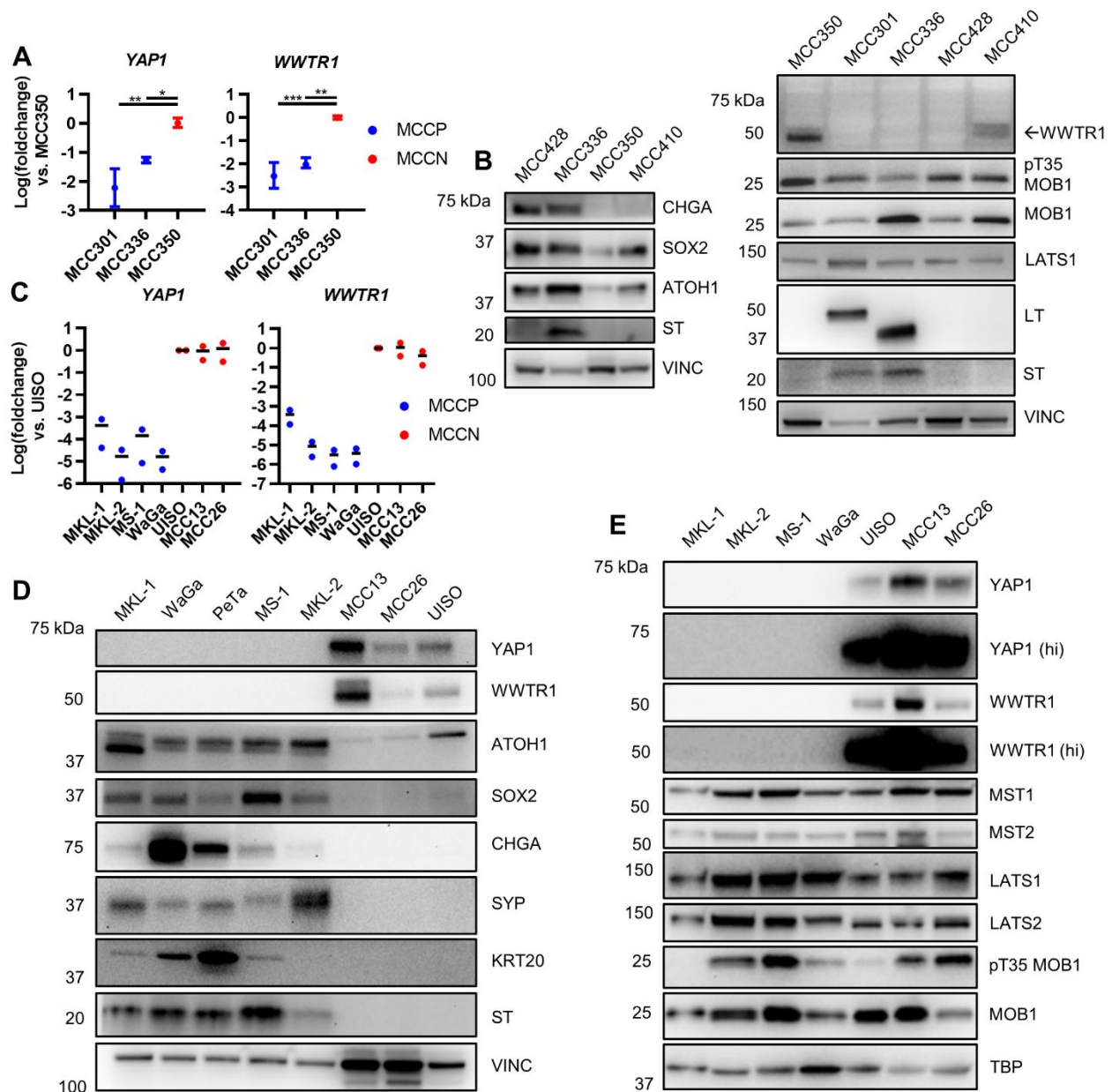


Figure 3.8. Analyses of *YAP1/WWTR1* expression in PDCLs and established MCC cell lines. A. RT-qPCR of *YAP1/WWTR1* in PDCLs. N = 3; mean + CI from technical replicate SD; normalized to 18S rRNA then MCC350; one-way ANOVA with Dunnett post-hoc tests; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. B. Immunoblots of *WWTR1*, NE markers, Hippo pathway kinase *LATS1*, and *MOB1* in PDCLs. N = 1. C. RT-qPCR of *YAP1/WWTR1* in established MCC cell lines. N = 2; mean shown; normalized to 18S rRNA then UISO. D. Immunoblot of established MCC cell lines for *YAP1/WWTR1* and NE markers. Representative of 3 images. E. Immunoblot of the Hippo pathway kinases and *MOB1* in established MCC cell lines. Representative of 2 images.

YAP1/WWTR1 and NE marker expression correlates with morphology of MCC cell lines

We noticed the transcriptional profiles of both the PDCLs and established cell lines were strongly correlated with morphology. Among the established cell lines the NE-hi lines formed suspended neurospheres, while the NE-lo lines were adherent (Figure 3.9A). While all PDCLs formed neurospheres, they displayed a spectrum of density and adhesiveness. The NE-hi lines MCC428, MCC301, and MCC336 formed loose and chain-like or sheet-like clumps, while the NE-lo lines MCC350 and MCC410 formed the densest and most difficult-to-dissociated clumps (Figure 3.9B).

Morphological plasticity was also observed in some of the PDCLs. Upon enzymatic dissociation during normal passaging, an adherent population of cells was isolated from suspended MCC516 neurospheres. We separately cultured the adherent (MCC516a) and suspension (MCC516s) populations for 63 days until both populations were harvested for scRNA-seq analysis (Figure 3.9C). The scRNA-seq resulted in 8,368 high-quality cells from MCC516s and 782 from MCC516a, and the data were integrated into a single clustering analysis to identify conserved transcriptional groupings between culturing conditions. Thirteen clusters were identified, most of which expressed the cluster 0 genes (Figure 3.9D, 3.9E). Importantly, we observed co-clustering transcriptional populations of some MCC516a and MCC516s cells. Furthermore, both MCC516a and MCC516s contained populations of *MKI67*-expressing cells in clusters 6 and 8, supporting the notion that both culturing methods resulted in proliferating cells (Figure 3.10A). Expression of *YAP1*, *WWTR1*, *CCN1*, and *CCN2* were observed within clusters 3 and 12, and these clusters were enriched for adherent rather than suspension cells (Figure 3.9F, 3.10B). Extraction of all *WWTR1*-positive cells again showed a negative correlation between *WWTR1*/*YAP1* and NE/cluster 0 gene expression (Figure 3.9G). Importantly, these *WWTR1*-positive cells were nearly exclusively adherent. However, *KRT8*-expressing *YAP1*/*WWTR1*-positive cells were observed, indicating these adherent, non-NE cells were indeed MCC. Additionally, a small number of *WWTR1*-positive cells were present in the NE-hi MCC516s population, albeit with reciprocally lower expression of *WWTR1*, identifying a potential source population for MCC516a.

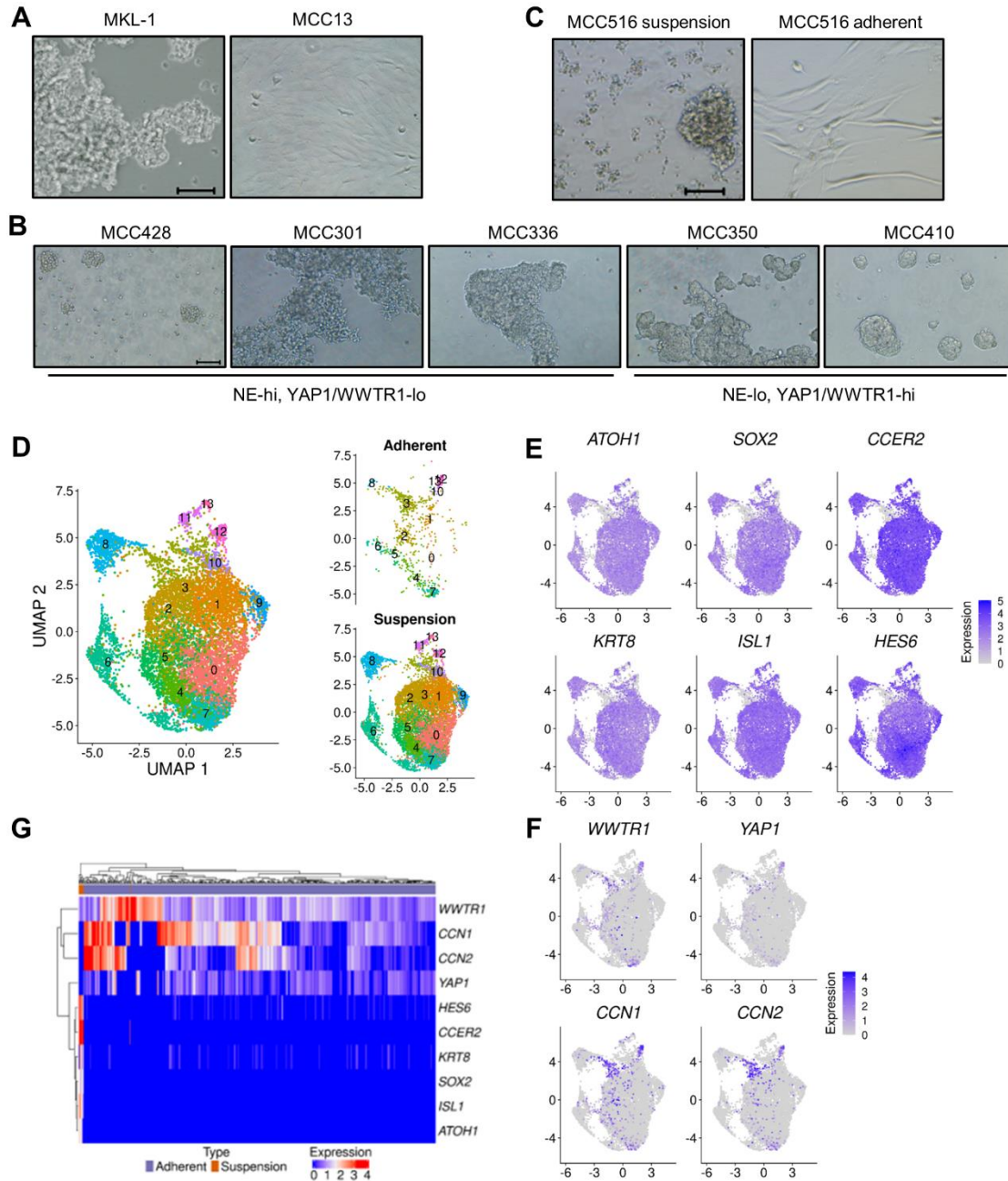


Figure 3.9. YAP1/WWTR1 and NE gene expression correlate with MCC cell line morphology. A. Morphologic comparison of MKL-1 and MCC13 established cell lines. B. Morphologic comparison showing that NE-lo, YAP1/WWTR1-hi PDCLs exhibit increased clumping versus NE-hi, YAP1/WWTR1-lo lines. C. Morphologic comparison between divergent populations of the PDCL MCC516. A, B, C all 10x magnification; scale bars 100 μ m. D. DimPlots of integrated scRNA-seq clustering analysis of two divergent suspension (MCC516s) and adherent (MCC516a) PDCLs derived from the same parent population. E. Feature plots of normalized expression of cluster 0 genes and F. YAP1, WWTR1, CCN1 and CCN2 in MCC516s/a. G. Heatmap of normalized expression of YAP1, WWTR1, CCN1, CCN2, and cluster 0 genes in each WWTR1-expressing cell (n = 446) from the MCC516s/a samples analyzed by scRNA-seq.

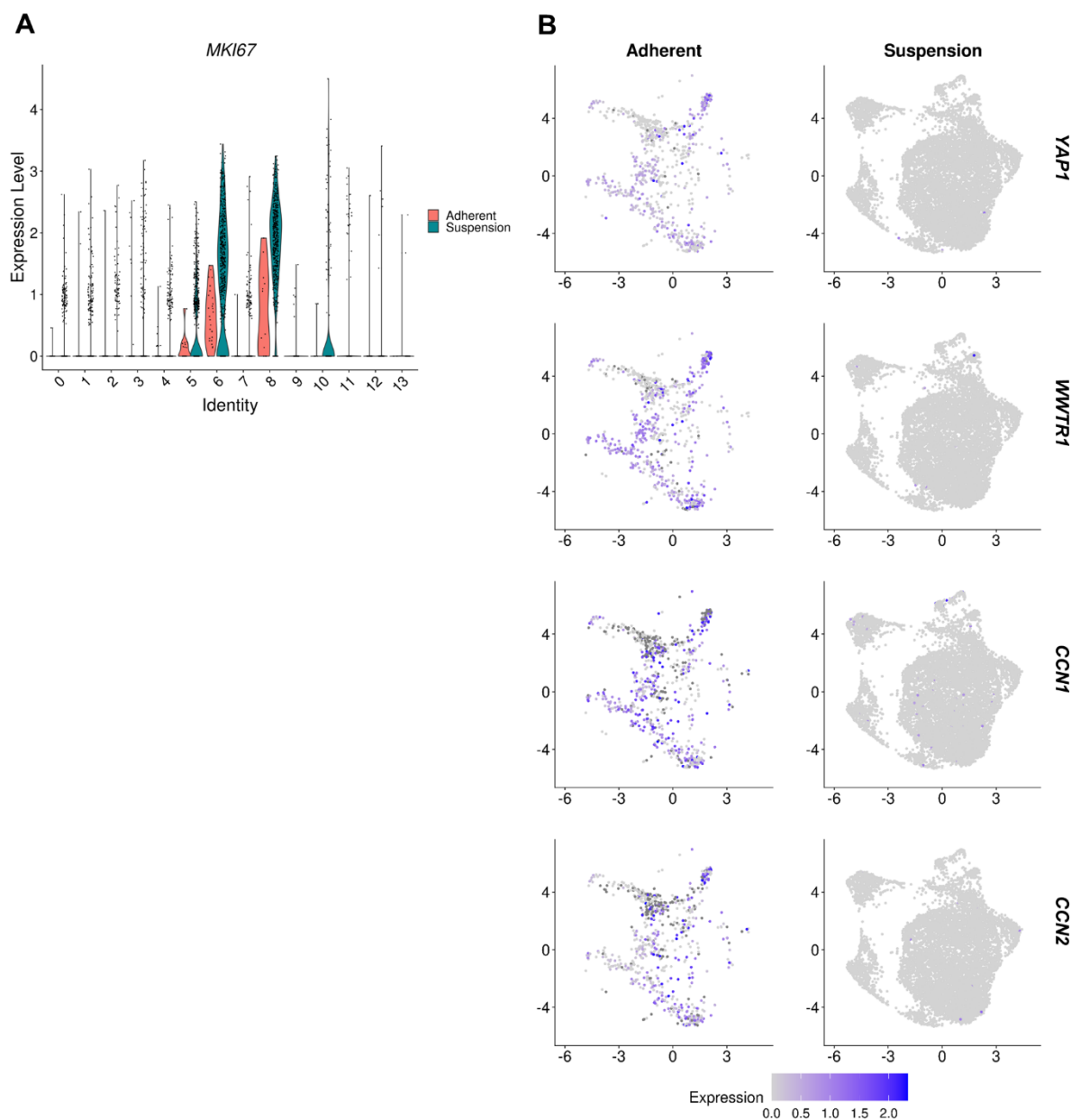


Figure 3.10. Additional scRNA-seq characterization of MCC516s/a. A. Violin plots of normalized *MKI67* expression in each cluster for MCC516s/a. B. Feature plots of normalized expression of *YAP1*, *WWTR1*, *CCN1*, and *CCN2* in MCC516s/a.

The NE-lo MCC subtype trends towards a higher recurrence rate

We examined patient metadata associated with the MCC tumor biopsies to determine if there was clinical relevance of the NE-hi and NE-lo transcriptional groups (Table 3.1, Figure 3.11). NE-hi and NE-lo transcriptional groups did not differ significantly in terms of overall survival (OS) or recurrence-free survival (RFS) (Figure 3.12A, 3.12B). However, reclassifying tumors based on viral-status revealed significantly decreased OS and RFS in patients with MCCN as compared to MCCP (Figure 3.13A, 3.13B). Combining the two classification schemes showed MCCP and MCCN tumors clustered together in terms of OS and RFS regardless of transcriptional group (Figure 3.14A, 3.14B). As such, NE-hi and NE-lo transcriptional groupings are minimally predictive of disease outcomes as compared to viral-status. YAP1 expression has been associated with recurrent disease in prostate cancer (Marx et al., 2020) and drug-resistant disease in hepatocellular, ovarian, pancreatic, and breast cancers (Nguyen and Yi, 2019). Interestingly, 72% (13/18) of NE-lo tumor biopsies came from patients who developed recurrent disease as compared to only 42% (10/23) of NE-hi (Table 3.1, Figure 3.11). However, due to the limited sample size these results are descriptive and not necessarily predictive.

Table 3.1. Patient demographics by NMF subgrouping

	NMF 1 (NE-lo) (N=18)	NMF 2 (NE-hi) (N=23)	Overall (N=41)
Age			
Mean (SD)	74.0 (8.33)	75.7 (13.3)	75.0 (11.3)
Median [Min, Max]	73.4 [58.3, 88.5]	75.8 [41.8, 94.7]	75.1 [41.8, 94.7]
Age (category)			
<73	9 (50.0%)	10 (43.5%)	19 (46.3%)
>= 73	9 (50.0%)	13 (56.5%)	22 (53.7%)
Gender			
Female	5 (27.8%)	9 (39.1%)	14 (34.1%)
Male	13 (72.2%)	14 (60.9%)	27 (65.9%)
Race			
White	18 (100%)	23 (100%)	41 (100%)
Site			
Chest/Abdomen/Pelvis	4 (22.2%)	4 (17.4%)	8 (19.5%)
Head and Neck	5 (27.8%)	3 (13.0%)	8 (19.5%)
Lower Extremities	6 (33.3%)	7 (30.4%)	13 (31.7%)
Upper Extremities	3 (16.7%)	5 (21.7%)	8 (19.5%)
Unknown	0 (0%)	4 (17.4%)	4 (9.8%)
Clinical AJCC stage			
1	2 (11.1%)	3 (13.0%)	5 (12.2%)
2	3 (16.7%)	1 (4.3%)	4 (9.8%)
3	13 (72.2%)	14 (60.9%)	27 (65.9%)
4	0 (0%)	5 (21.7%)	5 (12.2%)
Co-morbidities*			
Rheumatoid Arthritis	1 (5.6%)	1 (4.3%)	2 (4.8%)
Crohn's Disease	0 (0%)	1 (4.3%)	1 (2.4%)
Psoriasis	0 (0%)	1 (4.3%)	1 (2.4%)
Other cancer*			
Squamous Cell Carcinoma	6 (33.3%)	3 (13.0%)	9 (22.0%)
Basal Cell Carcinoma	2 (11.1%)	2 (8.7%)	4 (9.8%)
Melanoma	1 (5.6%)	1 (4.3%)	2 (4.9%)
Lymphoma	0 (0%)	1 (4.3%)	1 (2.4%)
Other	3 (16.7%)	2 (8.7%)	5 (12.2%)
MCPyV status			
Negative	8 (44.4%)	6 (26.1%)	14 (34.1%)
Positive	10 (55.6%)	17 (73.9%)	27 (65.9%)
Pre-first-biopsy treatment*			
Chemotherapy	6 (33.3%)	2 (8.7%)	8 (19.6%)
Immunotherapy	1 (5.6%)	1 (4.3%)	2 (4.9%)
Radiation	0 (0%)	1 (4.3%)	1 (2.4%)
Surgical Removal	0 (0%)	1 (4.3%)	1 (2.4%)
Post-first-biopsy treatment*			
Chemotherapy	9 (50.0%)	7 (30.4%)	16 (39.0%)

Table 3.1. Patient Demographics by NMF Subgrouping, Continued

Immunotherapy	11 (61.1%)	10 (43.5%)	21 (51.2%)
Radiation	3 (16.7%)	6 (26.1%)	9 (22.0%)
Recurrence			
No	5 (27.8%)	13 (56.5%)	18 (43.9%)
Yes	13 (72.2%)	10 (43.5%)	23 (56.1%)
Tobacco History			
Current	1 (5.6%)	1 (4.3%)	2 (4.9%)
Former	8 (44.4%)	9 (39.1%)	17 (41.5%)
Never	9 (50.0%)	13 (56.5%)	22 (53.7%)
Alcohol History			
Current	8 (44.4%)	9 (39.1%)	17 (41.5%)
Former	1 (5.6%)	2 (8.7%)	3 (7.3%)
Never	8 (44.4%)	12 (52.2%)	20 (48.8%)
Unknown	1 (5.6%)	0 (0%)	1 (2.4%)

*Patients may have multiple co-morbidities, other cancers, and treatments which may not add to 100%.

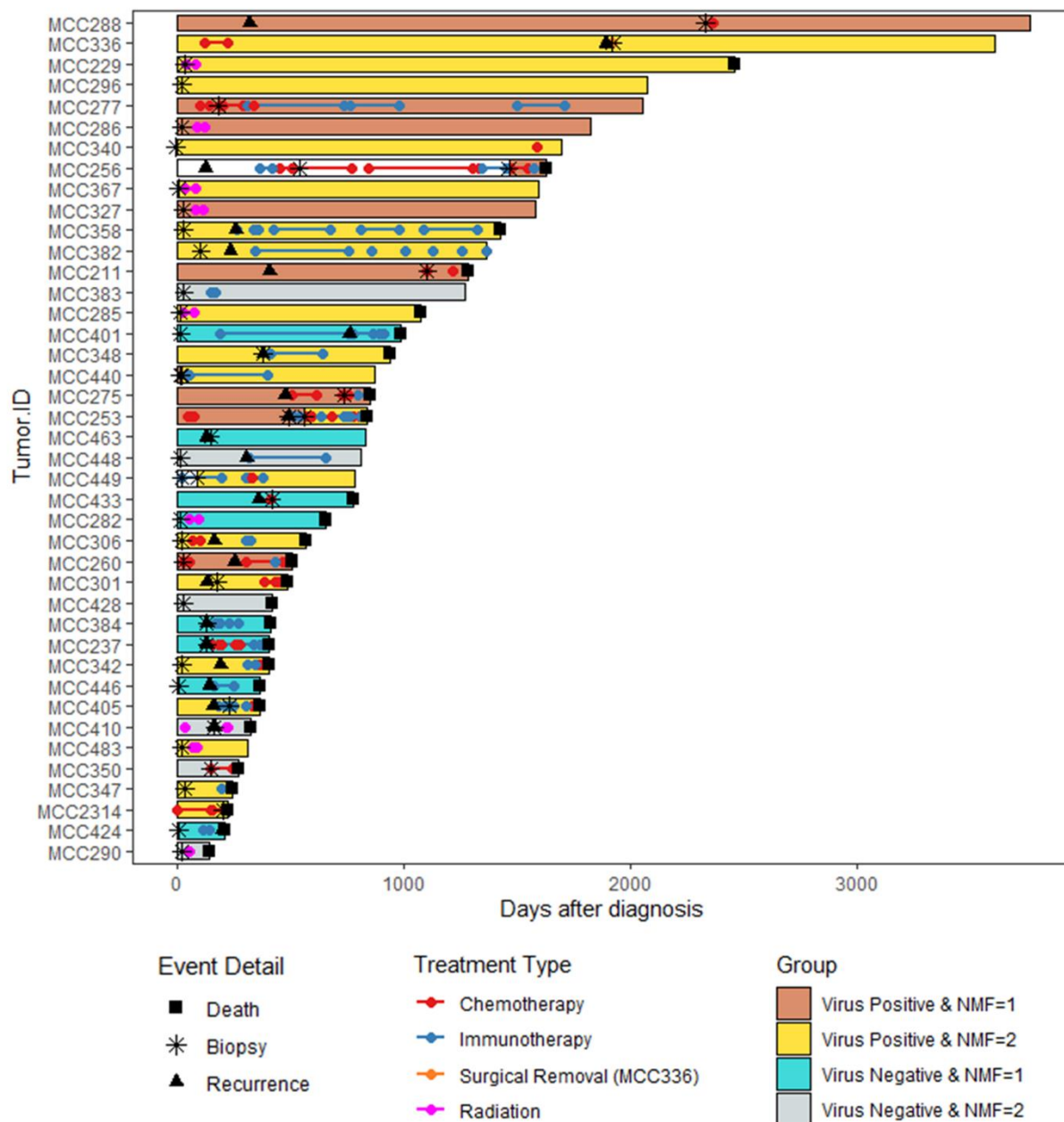
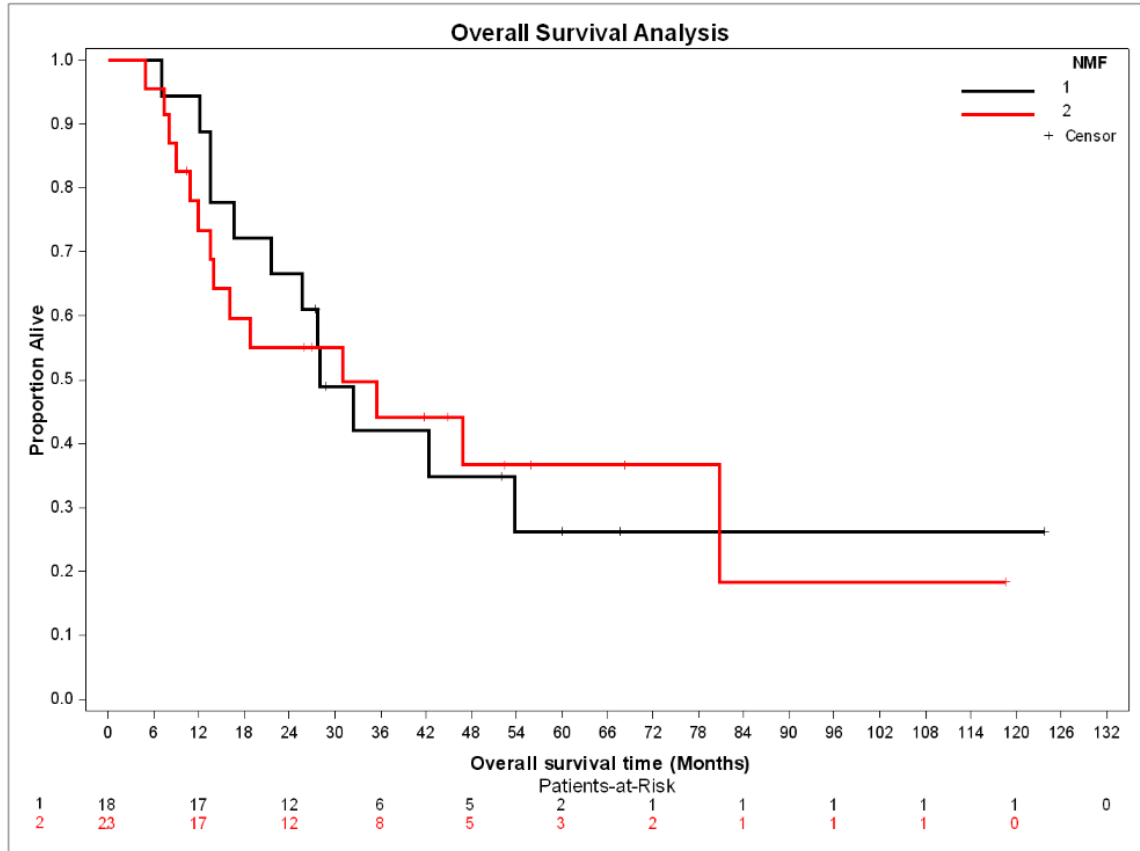


Figure 3.11. Longitudinal analysis of MCC patient metadata. Swimmer plot of selected patient metadata for 41 bulk tumor RNA-seqs with NMF- and MCPyV-defined subgroupings. White column sections indicate the sample was removed from the bulk RNA-seq analyses due to insufficient tumor. Four tumor biopsies present in the bulk RNA-seq represented recurrences of biopsies from the same patient. With respect to NMF grouping in this analysis, we only considered the first viable tumor biopsy as representative for the patient.

A.



B.

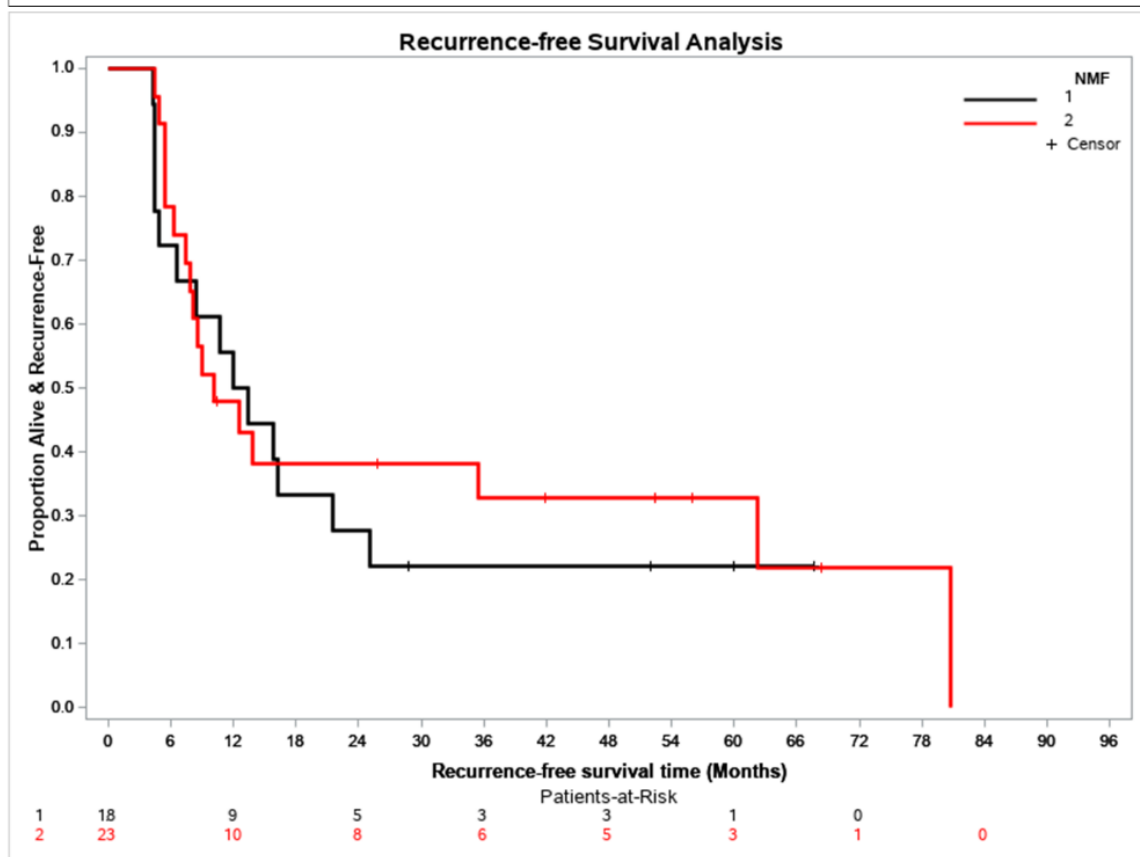
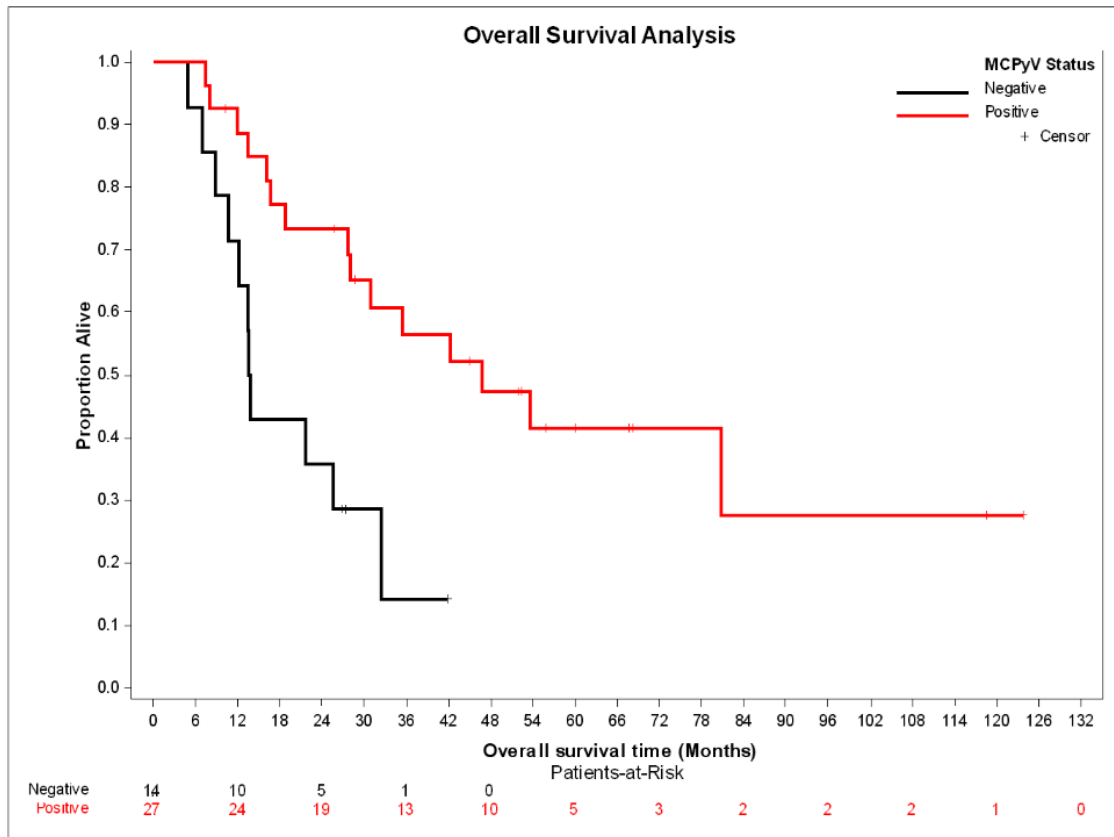


Figure 3.12. OS and RFS analyses of patient metadata from NMF-defined subgroupings. A. Kaplan-Meier (KM) OS curves of patient metadata stratified on NMF subgroups identified in the bulk tumor biopsy RNA-seq dataset. OS was measured from time of patient inclusion in the study until death or study termination. The median OS (95% CI) was 28.1 and 31 months for NMF group 1 and 2, respectively (KM method). At 60 months, the OS was 26% (95% CI 11-63%) and 37% (95% CI 20-67%) for NMF group 1 and 2 respectively (KM method). NMF group1 and 2 did not differ significantly in terms of OS (P-value = 0.7589, Logrank test, stratified by age (≥ 73 vs. <73)). B. KM PFS curves of patient metadata stratified on NMF subgroups identified in the bulk tumor biopsy RNA-seq dataset. PFS was measured from time of patient inclusion in the study until the first noted recurrence or study termination. The median PFS (95% CI) was 12.7 and 10.1 months for NMF group 1 and 2, respectively. At 60 months, the PFS was 22% (95% CI 9-53%) and 33% (95% CI 18-60%) for NMF group 1 and 2, respectively. NMF group 1 and 2 did not differ significantly in terms of PFS (P-value 0.7037; Logrank test, stratified by age(≥ 73 vs. <73)).

A.



B.

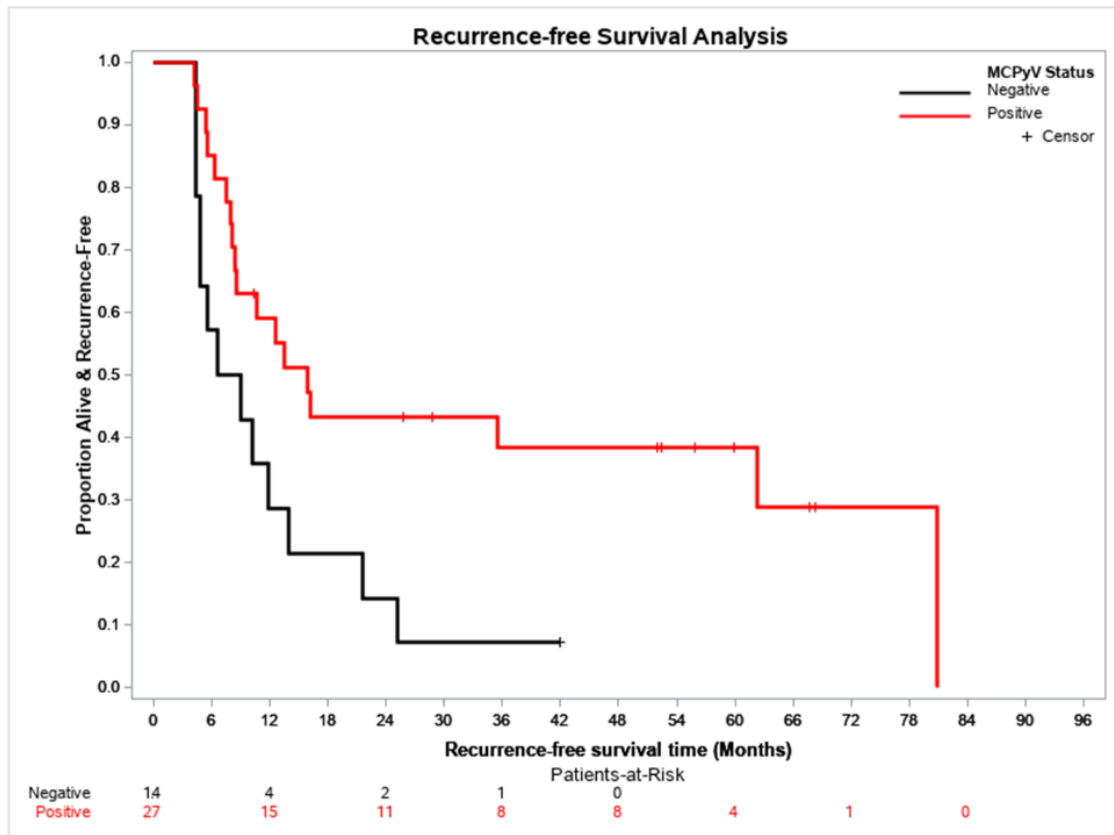
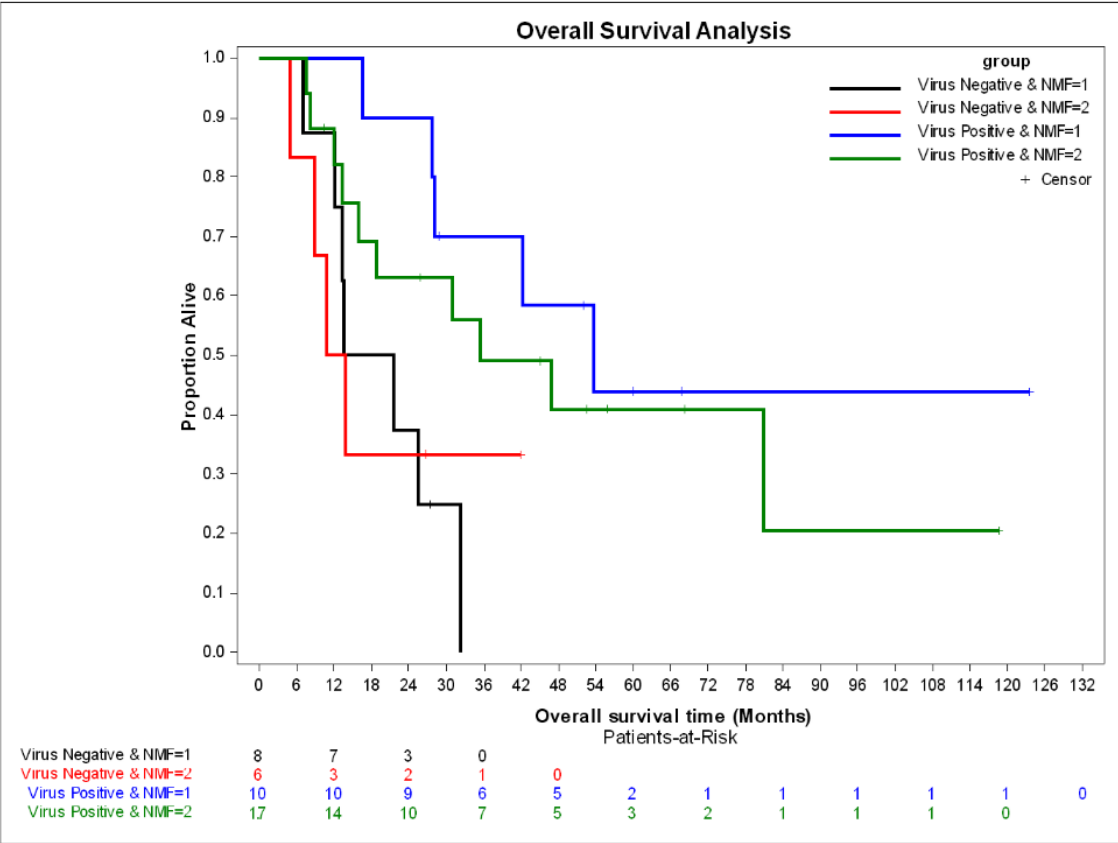


Figure 3.13. OS and RFS analyses of patient metadata from MCPyV-defined subgroupings.

KM OS curves of patient metadata stratified on MCPyV-status. OS was measured from time of patient inclusion in the study until death or study termination. The median OS (95% CI) was 13.7 and 46.8 months for MCPyV-negative and -positive groups, respectively (KM method). At 60 months, the OS was 0% (95% CI NE-NE) and 41% (95% CI 25-68%) for MCPyV-negative and -positive patients, respectively (KM method). MCPyV-positive patients had significantly increased OS as compared to MCPyV-negative (P-value = 0.0092, Logrank test, stratified by age (≥ 73 vs. <73)). B. KM PFS curves of patient metadata stratified on MCPyV-status. PFS was measured from time of patient inclusion in the study until the first noted recurrence or study termination. The median PFS (95% CI) was 7.8 and 15.8 months for MCPyV-negative and -positive samples, respectively. At 60 months, the PFS was 0% (95% CI NE-NE) and 38% (95% CI 23-63%) for MCPyV-negative and -positive patients, respectively. MCPyV-positive patients had significant increased PFS as compared to MCPyV-negative (P-value 0.0262; Logrank test, stratified by age (≥ 73 vs. <73)).

A.



B.

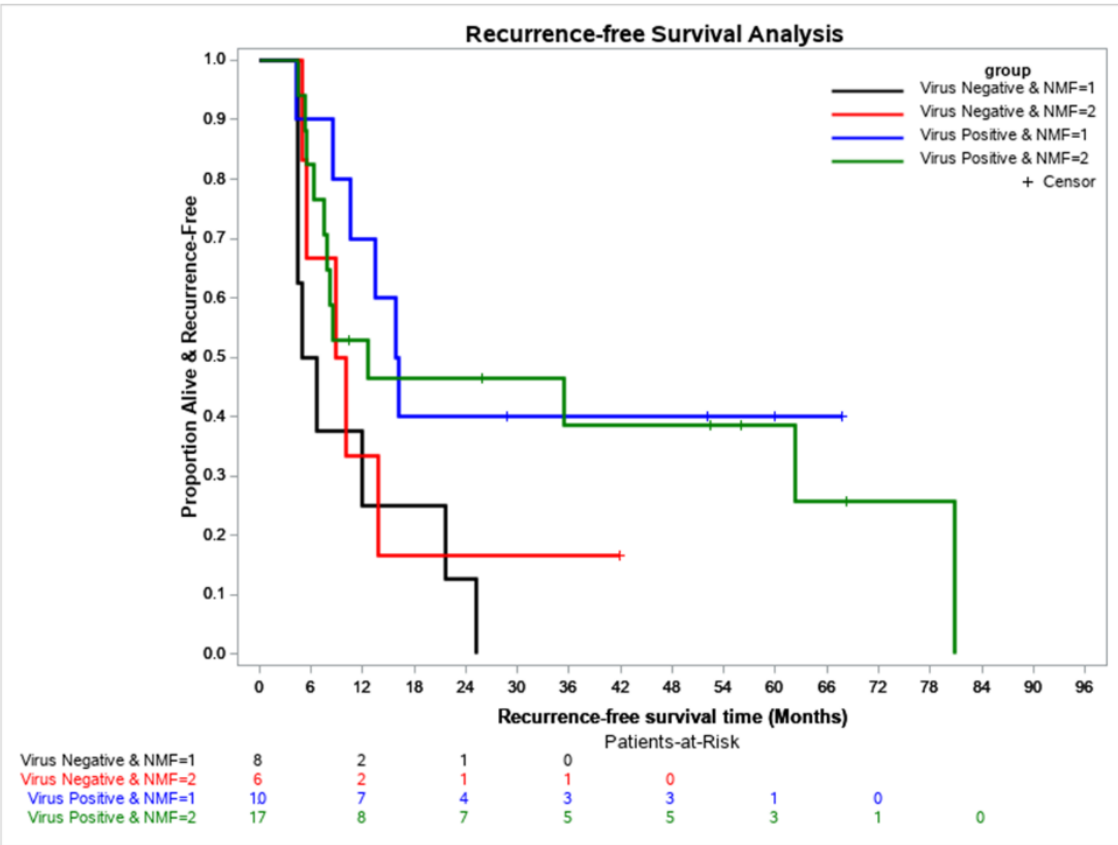


Figure 3.14. OS and RFS analyses of patient metadata from combination of NMF- and MCPyV-defined subgroupings. KM OS curves of patient metadata stratified on NMF-group and MCPyV-status. OS was measured from time of patient inclusion in the study until death or study termination. The median OS (95% CI) was 17.6 (NMF1/MCCN), 12.3 (NMF2/MCCN), 53.6 (NMF1/MCCP), and 35.5 (NMF2/MCCP) months. At 60 months (95% CI), the OS was 0% (NMF1/MCCN; NE-NE), 0% (NMF2/MCCN; NE-NE), 44% (NMF1/MCCP; 20-96%), and 41% (NMF2/MCCP; 22-76%). There was no significant interaction between NMF- and MCPyV-subgroups in terms of OS (P-value = 0.0668, Logrank test, stratified by age (≥ 73 vs. <73)). B. KM PFS curves of patient metadata stratified on NMF-group and MCPyV-status. PFS was measured from time of patient inclusion in the study until the first noted recurrence or study termination. The median PFS (95% CI) was 5.7 (NMF1/MCCN), 9.5 (NMF2/MCCN), 16.0 (NMF1/MCCP), and 12.5 (NMF2/MCCP) months. At 60 months (95% CI), the PFS was 0% (NMF1/MCCN; NE-NE), 0% (NMF2/MCCN; NE-NE), 40% (NMF1/MCCP; 19-85%) and 39% (NMF2/MCCP; 21-73%). There was no significant interaction between NMF- and MCPyV-subgroups in terms of PFS (P-value = 0.1329, Logrank test, stratified by age (≥ 73 vs. <73)).

YAP1/WWTR1 expression in NE-hi MCCP cells induces TEAD-dependent growth-suppressive transcriptional changes

Expression of YAP1/WWTR1 in *YAP1/WWTR1*-silenced cancers, including NE-hi SCLC, can suppress proliferation (Cottini *et al.*, 2014; Grieve *et al.*, 2019; Nishikawa *et al.*, 2011; Pearson *et al.*, 2021). To determine if induction of YAP1/WWTR1 can suppress proliferation in NE-hi MCCP, we transduced MKL-1 cells with doxycycline (DOX)-inducible wildtype or mutant YAP or WWTR1, or GFP control. The YAP1/WWTR1 mutants included transcriptionally hyperactive YAP1 5SA (Zhao *et al.*, 2010) and WWTR1 4SA (Lei *et al.*, 2008), TEAD binding-defective mutants YAP1 S94A (Zhao *et al.*, 2008) and WWTR1 F52A (Chan *et al.*, 2009; Kofler *et al.*, 2018), as well as constructs combining both types of mutations (YAP1 6SA and WWTR1 4SA/F52A). Upon induction, the wildtype and hyperactive but not TEAD binding-defective or dual-mutant constructs increased expression of the YAP1 and WWTR1 direct transcriptional target, *CCN1* (Figure 3.15A; Figure 3.16A). Induction of wildtype and hyperactive YAP1 significantly decreased cell viability relative to GFP compared to either TEAD-binding defective or dual-mutant YAP1 (Figure 3.17A). However, only induction of hyperactive WWTR1 was able to significantly reduce viability relative to TEAD-binding defective or dual-mutant WWTR1. Interestingly, expression of wildtype or hyperactive YAP1 or WWTR1 but not TEAD-binding defective or dual-mutants led to the formation of dense, difficult to dissociate neurospheres that were similar in nature to the WWTR1-expressing PDCL MCC410 (Figures 3.9B, 3.15B, 3.16B).

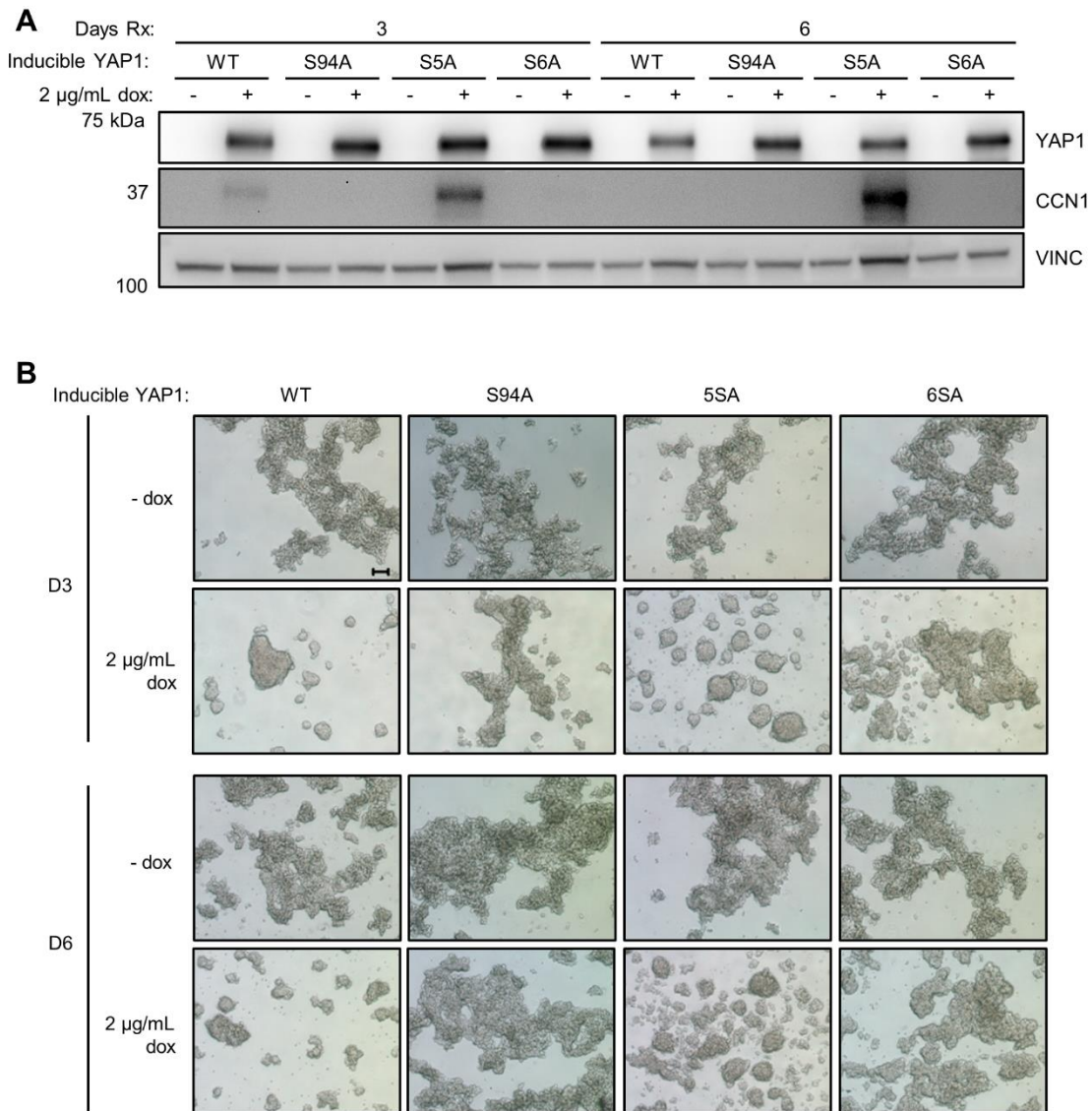


Figure 3.15. Transcriptionally active YAP1 expression in NE-hi MCCP cells induces CCN1 and adhesion-related morphological changes. A. Immunoblots of MKL-1 cells induced to express YAP1 for 3 or 6 days. Representative of 3 images. B. Bright-field image of MKL-1 cells upon expression of wildtype or mutant YAP1 for 3 or 6 days. 4x magnification; scale bar 100 μ m. Representative of 3 experiments.

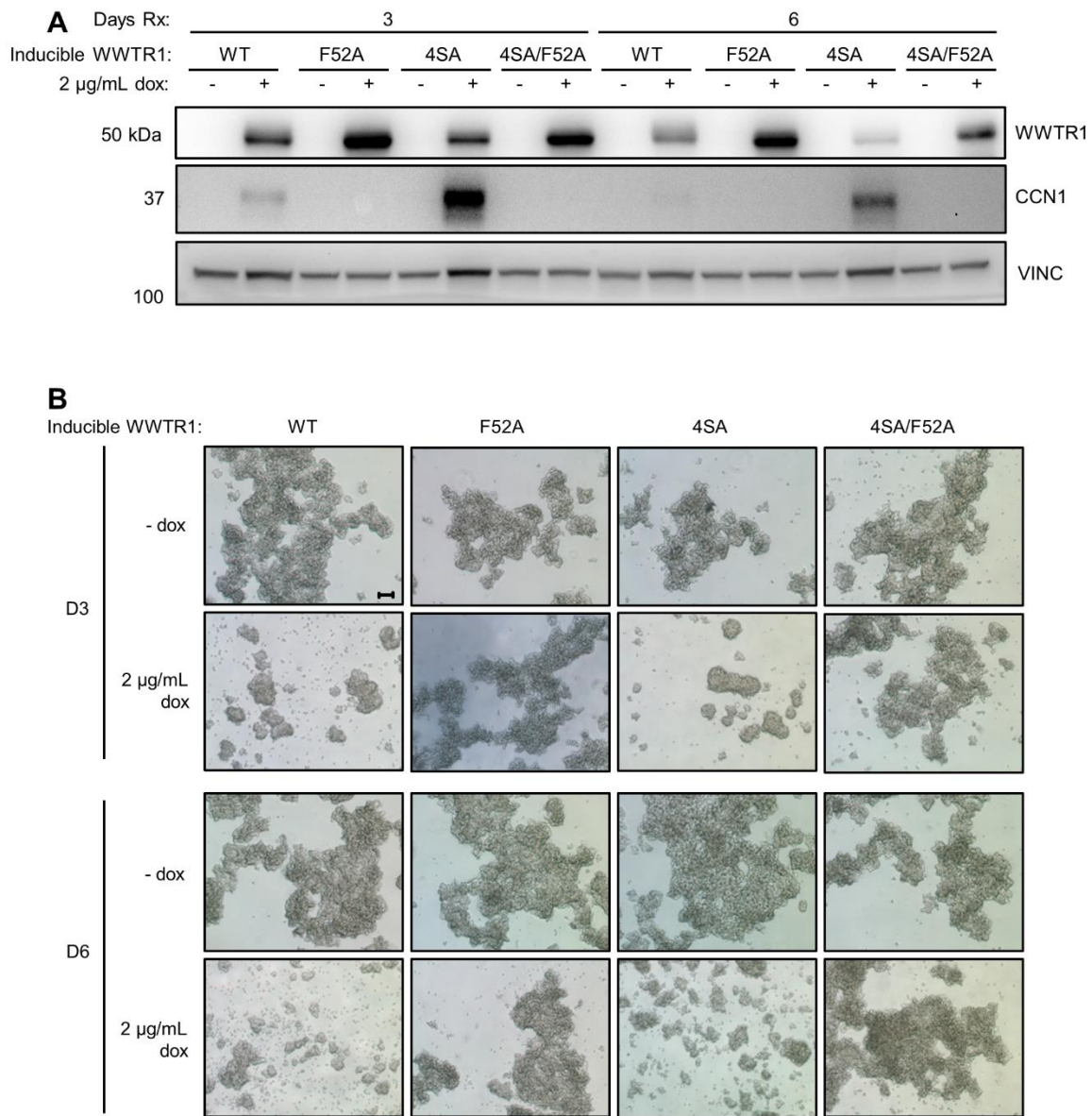


Figure 3.16. Transcriptionally active WWTR1 expression in NE-hi MCCP cells induces CCN1 and adhesion-related morphological changes. A. Immunoblots of MKL-1 cells induced to express WWTR1 for 3 or 6 days. Representative of 3 images. B. Bright-field image of MKL-1 cells upon expression of wildtype or mutant WWTR1 for 3 or 6 days. 4x magnification; scale bar 100 µm. Representative of 3 experiments.

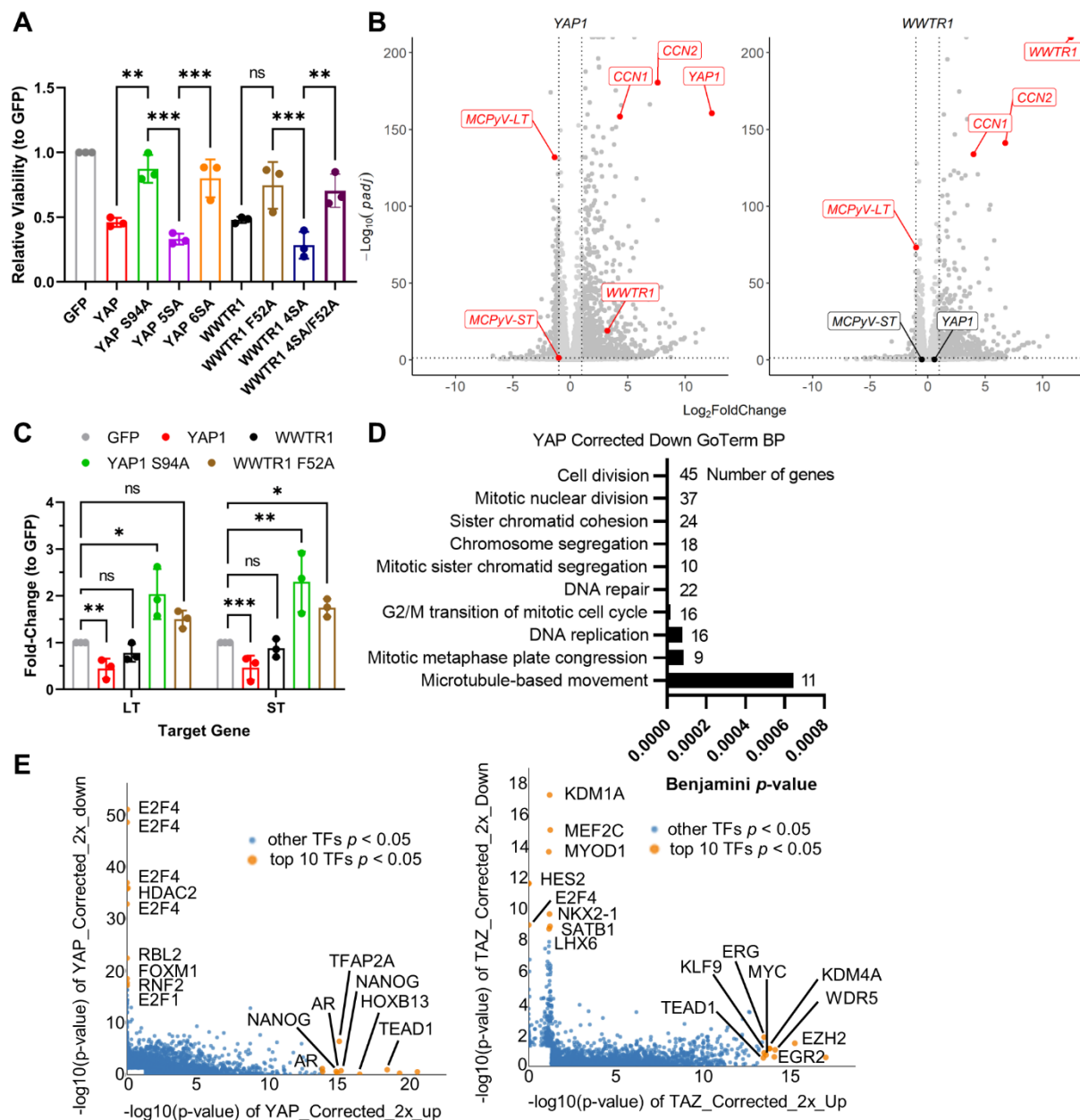


Figure 3.17. Expression of YAP1 or WWTR1 in NE-hi MCPyV cells causes growth-suppressive, TEAD-dependent transcriptional changes. A. CellTiter-Glo viability assay of MKL-1 cells inducibly expressing wildtype or mutant YAP1 or WWTR1 for 6 days. N = 3; mean + SD; 2-way repeated-measures ANOVA with Tukey post-hoc tests; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. B. Volcano plots highlighting DEG (red) in YAP1 or WWTR1 versus GFP conditions. Vertical dotted lines indicate Log₂FoldChange > 1 or < -1. Horizontal dotted line indicates $padj < 0.05$. C. RT-qPCR of MCPyV LT and ST levels after induction of wildtype or mutant YAP1 or WWTR1 for 3 days. N = 3; mean + SD; normalized to 18S rRNA then to GFP and presented as Fold-Change versus GFP; one-way ANOVA with Dunnett post-hoc tests was performed on ΔCt values normalized to 18S rRNA; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. D. GoTerm Biological Process (BP) analysis of two-fold downregulated DEG in corrected YAP dataset. E. LISA of corrected two-fold up- and down-regulated DEG in the YAP1- and WWTR1-expressing conditions.

Since only the induction of transcriptionally active YAP1 or WWTR1 induced proliferative loss in NE-hi MCCC, we performed transcriptome profiling to identify potential downstream effectors. As expected, previously published direct YAP1/WWTR1 target genes (Wang *et al.*, 2018) were broadly induced in the wildtype and not TEAD-binding defective mutant samples (Figure 3.18A). We defined differentially expressed genes (DEG) as those with an absolute $\log_2$ -fold change cutoff of ≥ 1 and $padj \leq 0.05$, as compared to GFP control (Supplemental Tables 9-12). The DEG from wildtype YAP1 and WWTR1 were subtracted from their respective TEAD-binding defective mutants to identify genes specifically transcriptionally regulated by these complexes ("corrected targets"; Figure 3.18B; Supplemental Table 14). Strikingly, among the most significantly downregulated genes in both the corrected YAP1 and WWTR1 targets was MCPyV *LT*; MCPyV *ST* was also downregulated (Figure 3.17B). Since MCPyV *LT* is responsible for overcoming RB1-dependent G1/S cell cycle arrest, we reasoned *LT* downregulation may explain the YAP1/WWTR1-mediated proliferative defect. We validated the transcriptional repression of MCPyV *LT* and *ST* transcripts upon YAP1/WWTR1 induction by RT-qPCR (Figure 3.17C). In agreement with the RNA-seq, we observed a consistent and significant depletion of MCPyV *LT* and *ST* transcripts upon YAP1 induction as measured by RT-qPCR. WWTR1 was broadly less effective at modulating transcript levels (up or down) than YAP1 (Figure 3.17B). In line with this, we observed a smaller, albeit non-significant decrease in MCPyV *LT* and *ST* transcripts by RT-qPCR upon WWTR1 induction (Figure 3.17C). Expression of transcriptionally inactive YAP1 or WWTR1 led to modestly increased MCPyV *LT* and *ST* transcript levels. To identify gene sets significant enriched or depleted upon YAP1/WWTR1 induction we used DAVID (Huang *et al.*, 2009a; b) to perform a GOTerm analysis with the corrected DEG (Figure 3.17D). Upregulated gene sets for both YAP1 and WWTR1 were related to extracellular matrix organization and cell adhesion (Figure 3.18C, 3.18D; Supplemental Table 16). Indeed, these included many proteins involved in extracellular matrix organization and signal transduction including several integrin and collagen subunits (Figure 3.18E). Interestingly, we found that genes transcriptionally downregulated by YAP1 were significantly enriched for GOTerms involving DNA replication, chromosome segregation, and, more broadly, cell division (Figure 3.17D). We did not observe a significant enrichment for gene sets downregulated by WWTR1, in line with its more moderate effect on cell viability and transcription (Figure 3.17A, 3.17B). We performed epigenetic

Landscape *in Silico* deletion Analysis (LISA; (Qin et al., 2020)) on the corrected up- and downregulated DEG to predict regulating transcription factors based on publicly available ChIP-seq datasets (Figure 3.17E). As expected, TEAD1 was one of the most significant hits for the corrected YAP1 and WWTR1 upregulated gene lists. For the corrected, downregulated YAP1 but not WWTR1 targets we identified targets of critical cell-cycle regulators FOXM1 (Koo et al., 2012) and E2F (Chen et al., 2009). Together, these data imply that the viability decreases in NE-hi MCCP upon induction of YAP1/WWTR1 may act through TEAD-dependent transcriptional repression of MCPyV *LT* and subsequent RB1-dependent enforcement of G1/S arrest.

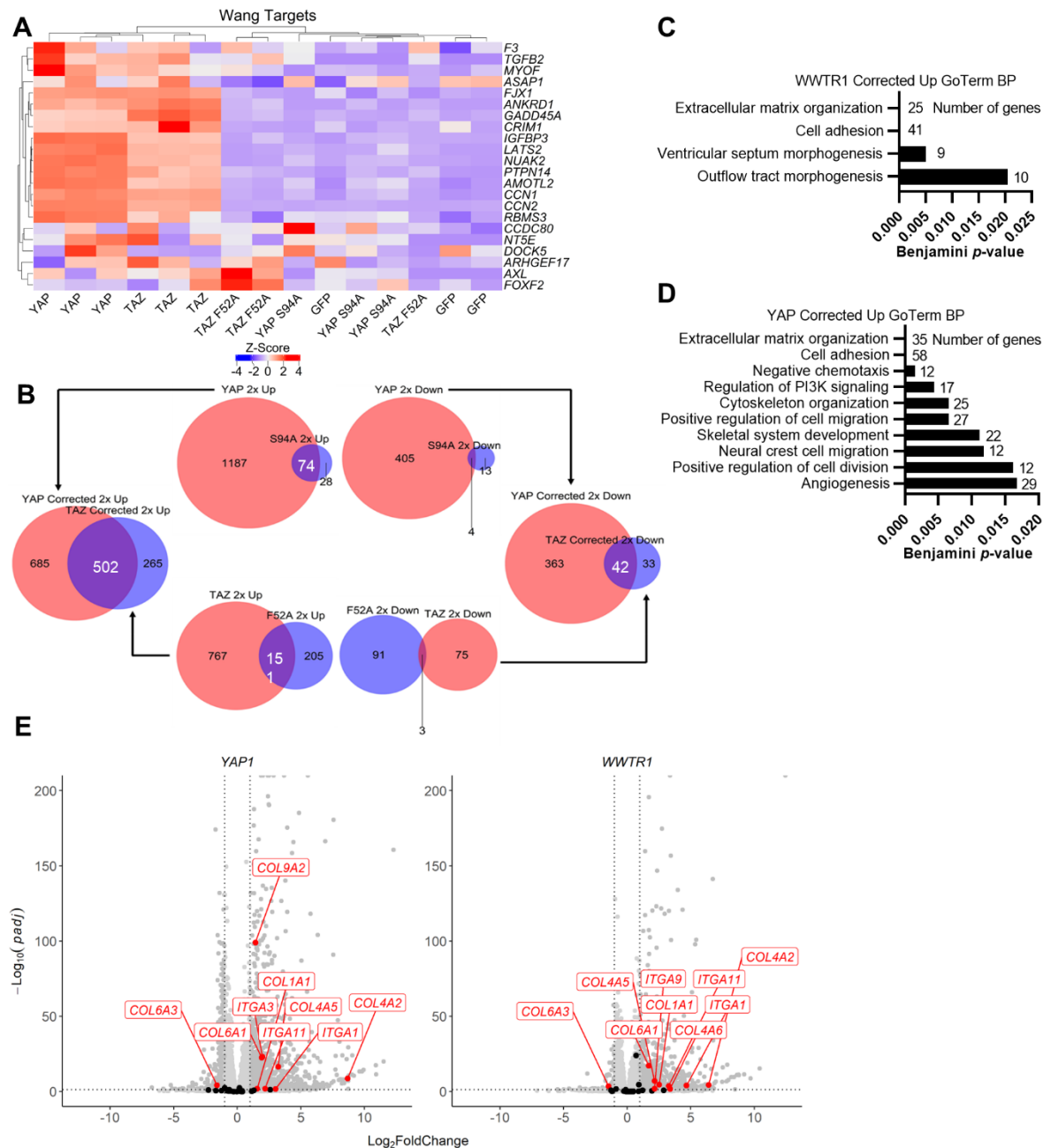
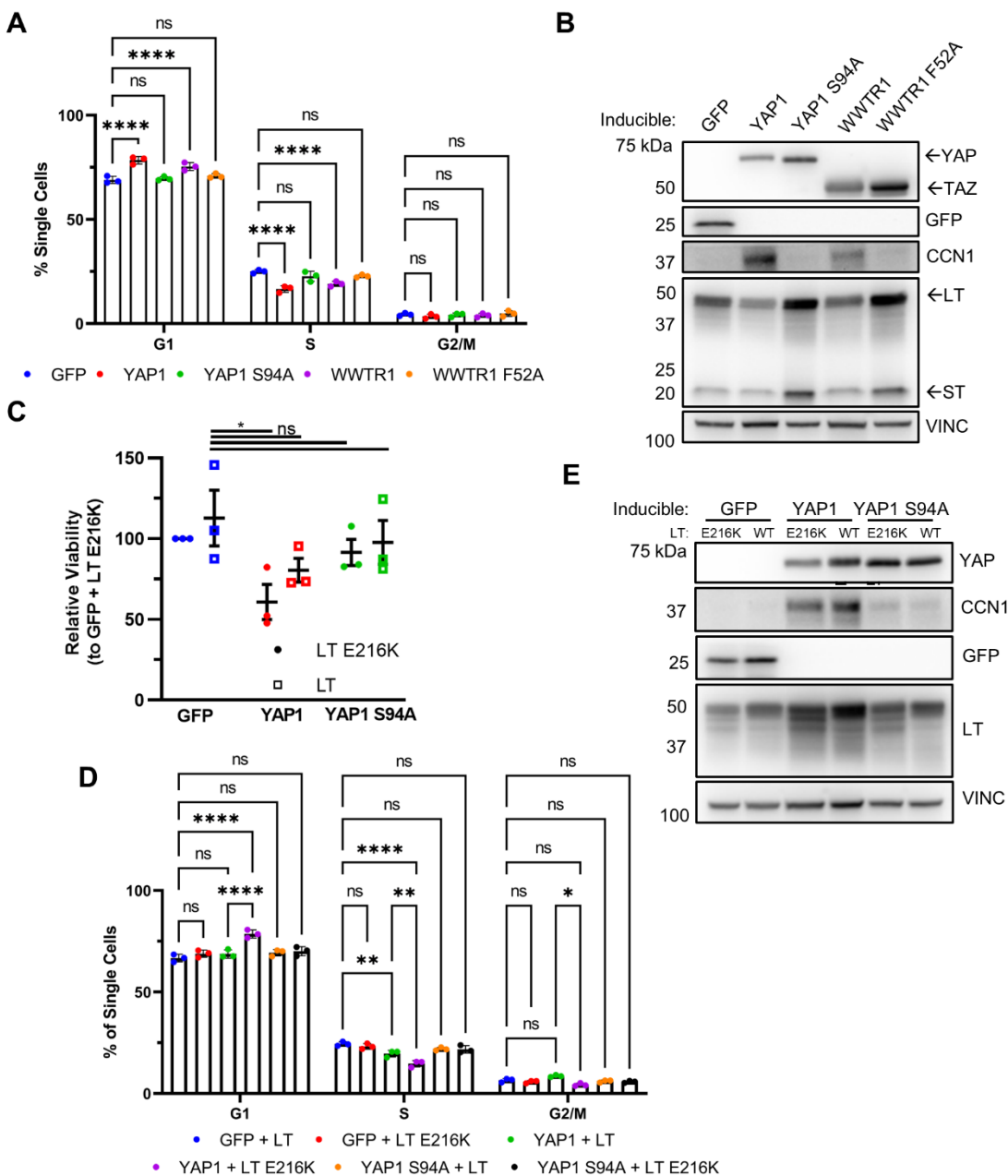


Figure 3.18. Quality control and correction strategy for RNA-seq of MKL-1 cells expressing YAP1 or WWTR1. A. Heatmap of high-confidence YAP1/WWTR1 direct target genes (42) in each expression condition. B. Venn diagrams showing “correction” scheme to obtain DEG whose expression changes were TEAD-dependent following induction of YAP1 or WWTR1. C. GoTerm BP analysis of two-fold upregulated DEG in corrected WWTR1 and B. YAP1 datasets. E. Volcano plot of the expression of integrin (ITG) and collagen (COL) subunits upon YAP1 or WWTR1 induction.

Expression of YAP1 suppresses cell-cycle progression in NE-hi MCCC cells through indirect downregulation of MCPyV LT expression

We explored whether expression of YAP1/WWTR1 in NE-hi MCCC cells induced cell-cycle arrest through suppression of MCPyV LT protein levels. Expression of wildtype but not TEAD-binding defective YAP1 S94A or WWTR1 F52A significantly decreased the percentage of cells in S-phase, while increasing those in G1 (Figure 3.19A). No effect on G2/M cells was noted. Immunoblot analysis confirmed downregulation of MCPyV LT protein levels upon induction of YAP1 or WWTR1 but not YAP1 S94A or WWTR1 F52A (Figure 3.19B). This YAP1/WWTR1 transcription-dependent decrease in MCPyV LT protein levels was also observed in an additional NE-hi MCCC cell line containing an independent MCPyV integration site (Figure 3.20A). In line with our RT-qPCR results (Figure 3.17C), we observed an increase in MCPyV LT protein levels upon expression of YAP1 S94A or WWTR1 F52A (Figure 3.19B). However, this effect was not conserved between the NE-hi MCCC cell lines assessed (Figure 3.20A). These data support the notion that suppression of MCPyV LT protein levels in YAP1- or WWTR1-expressing MCCC cells may induce RB1-dependent cell-cycle dysregulation.

To directly assess the requirement of LT-dependent RB1 suppression for YAP1- or WWTR1-mediated cell-cycle dysregulation, we transduced MKL-1 cells containing inducible wildtype or TEAD-binding defective YAP1 with constitutively active expression of MCPyV LT (L21; (Cheng *et al.*, 2013)) or MCPyV LT with a mutated LXCXE motif (E216K) previously reported to abrogate RB1 binding (Shuda *et al.*, 2008). Constitutive expression of wildtype but not RB1-binding defective MCPyV LT led to a partial but significant rescue of YAP1-dependent viability (Figure 3.19C) and S-phase population loss (Figure 3.19D). However, it did not alter the appearance of the TEAD-dependent morphological changes (Figure 3.20B). Immunoblot analysis verified that MCPyV LT protein levels were not suppressed in the constitutively expressing cells and CCN1 protein levels were increased following YAP1 induction (Figure 3.19E). These data indicated that TEAD-dependent loss of MCPyV LT protein levels induced RB1-dependent cell-cycle dysregulation upon YAP1 expression in NE-hi MCCC.



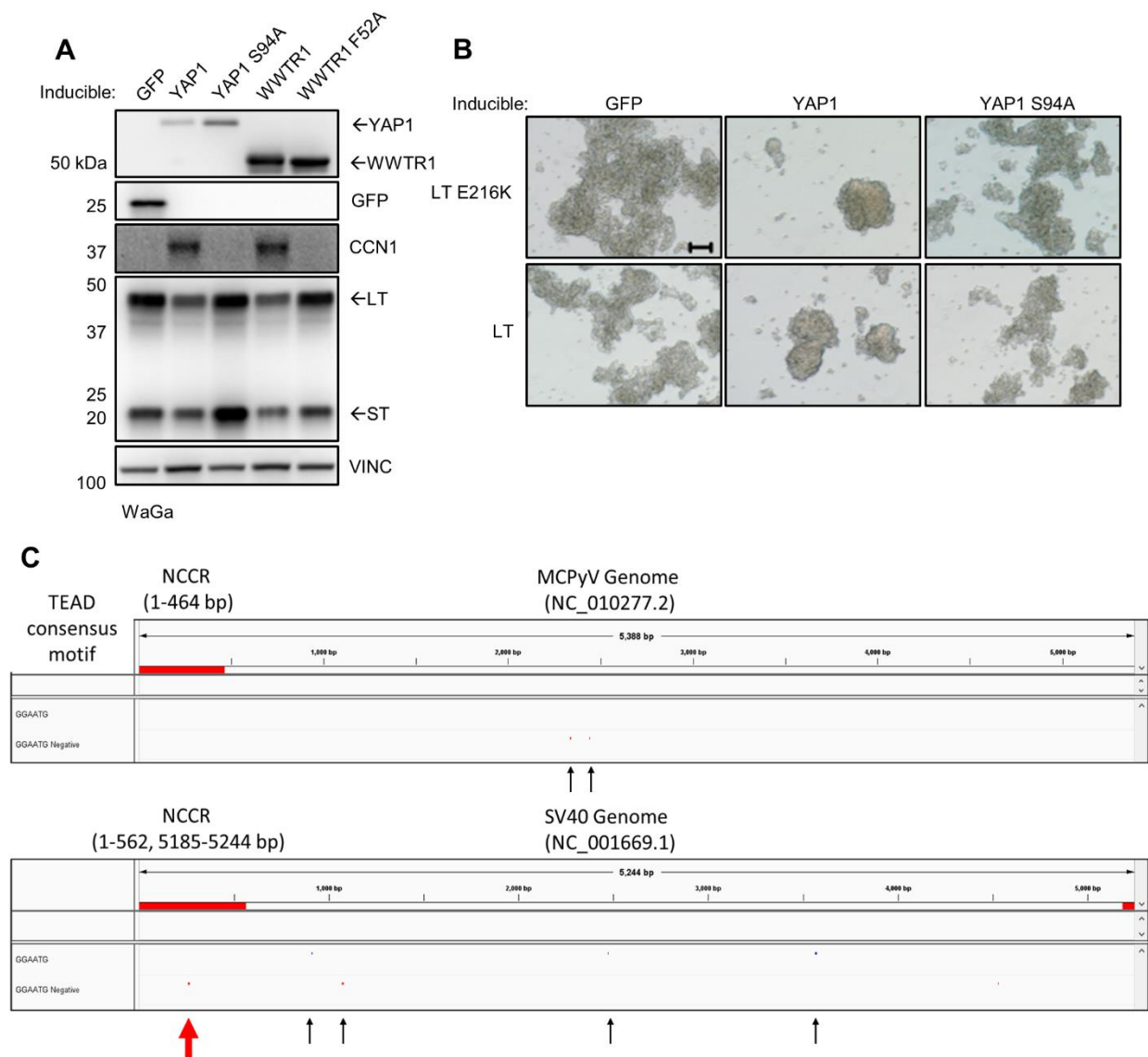


Figure 3.20. Additional analyses of YAP1/WWTR1 expression in MKL-1 and WaGa cells. A. WaGa cells induced to express wildtype YAP1 or WWTR1 for 3 days. Representative of 3 images. B. Bright-field microscopy of MKL-1 cells with inducible wildtype or mutant YAP1 and constitutive expression of wildtype or RB1-binding defective MCPyV LT induced for 6 days. 4x magnification; scale bar 100 μ m. Representative of 3 experiments. C. Integrative Genomics Viewer (IGV) plot of MCPyV (NC_010277.2) or SV40 (NC_001669.1) genomes highlighted for TEAD-consensus motif (5'-GGAATG-3'). Red bar represents NCCR for either MCPyV or SV40 genomes. Black arrows represent presence of TEAD-motif outside of NCCR, red arrows represent TEAD-binding motif within the NCCR.

Despite the transcription-dependent depletion of MCPyV LT levels by YAP1/WWTR1, it was unclear if these effects were due to direct regulation of the integrated MCPyV genome by the YAP1/WWTR1-TEAD transcriptional complex. The related polyomavirus, simian virus 40 (SV40), contains a consensus TEAD-binding motif (5'-GGAATG-3') in its non-coding control region (NCCR) which has been demonstrated to directly bind TEAD-containing transcriptional complexes (Davidson et al., 1988; Lin et al., 2017). As such, we assessed the MCPyV (RefSeq: NC_010277.2) and SV40 (RefSeq: NC_001669.1) NCCRs (red) for potential YAP1/WWTR1-TEAD binding motifs (Figure 3.20C). As expected, the TEAD-binding consensus motif was present in the NCCR of the SV40 genome. Importantly, the MCPyV NCCR lacked a TEAD-binding motif, indicating that YAP1/WWTR1 are unlikely to directly bind and regulate expression of MCPyV T antigens. As a result, the effects of YAP1/WWTR1 on MCPyV LT protein- and RNA-levels are likely indirect.

Discussion

MCC is an aggressive NE skin cancer currently divided into two etiological subtypes defined by clonally integrated MCPyV or UV-induced mutagenesis. Despite profound genetic differences, histological marks do not differ between subtypes. Prior work has shown variable expression of NE genes in MCC (Gravemeyer *et al.*, 2021), while studies of other NE cancers including SCLC used NE transcription factors as means of subtype deconvolution (Horie *et al.*, 2016; Ireland *et al.*, 2020; Ito *et al.*, 2016; McColl *et al.*, 2017; Nishikawa *et al.*, 2011; Pearson *et al.*, 2021). As such, we wondered if additional MCC subtypes could be identified thorough analysis of expressional datasets. Here we characterized transcriptional profiles of MCC tumor biopsies, PDCLs, and established cell lines and identified *de novo* a spectrum of NE transcription factor expression that is negatively correlated with expression of YAP1/WWTR1. Our major findings indicated: 1) MCCP and MCCN transcriptional profiles broadly overlap; 2) MCC can be classified according to bulk and single cell-level reciprocal expression of NE markers and YAP1/WWTR1; 3) a spectrum of NE and YAP1/WWTR1 gene expression is present within MCC, independent of viral-status; 4) NE-hi tumors contain small subpopulations of YAP1/WWTR1 expressing cells that can be sub-cultured; 5) YAP1/WWTR1 expression are strongly correlated with MCC cell morphology and influence cell-cell adhesiveness; and 6) YAP1/WWTR1 expression in NE-hi MCCP cells inhibits cell-cycle progression through indirect MCPyV LT suppression.

Our finding that the cluster 0/NE markers and YAP1/WWTR1 expression-based classification scheme is strongly correlated with cell morphology contextualizes earlier reports classifying MCC cell lines as “classic” or “variant”. Classic MCC has been characterized by higher expression of NE genes and growth in suspension while variant by lower NE gene expression and adherent growth (Leonard *et al.*, 1993; Leonard *et al.*, 1995; Van Gele *et al.*, 2004). However, prior studies have cast doubt on how well the variant cell lines represented MCC tumors (Daily *et al.*, 2015; Gravemeyer *et al.*, 2021). Our findings unify these observations upon reclassification of the classic and variant cell lines into NE-hi and NE-lo subgroups, respectively. Furthermore, other YAP1/WWTR1-silenced cancers typically grow in suspension while YAP1/WWTR1-expressing grow adherently (Cottini *et al.*, 2014; Grieve *et al.*, 2019; Horie *et al.*, 2016; Nishikawa *et al.*, 2011). We observed these effects in real-time upon isolation of an adherent MCC PDCL derived from an otherwise suspension culture. Importantly, scRNA-seq analysis of these patient-

derived cells revealed those growing adherently had higher expression of YAP1/WWTR1 and reciprocally lower NE gene expression than those in suspension. In line with this observation, siRNA-mediated depletion of YAP1 in an adherent YAP1-hi SCLC line induced a morphological phenotype similar to NE-hi, suspension SCLC cell lines (Horie *et al.*, 2016). Taken together, our work supports a model where the established MCC cell lines represent a spectrum of NE gene expression and that YAP1/WWTR1 are key determinants of cell line adherent or suspension morphology.

Our work has shown that YAP1/WWTR1 expression influences the cell-cell adhesiveness and density of MCC cell lines and neurospheres. Indeed, overexpression of YAP1/WWTR1 in a *YAP1/WWTR1*-silenced NE-hi suspension cell line induced formation of dense, difficult to dissociate neurospheres concurrent with the induction of integrin and collagen subunits. Overexpression of collagen has been shown to increase the rigidity of the extracellular matrix (ECM) resulting in overall tumor tissue stiffening and enhancing interactions with integrins to promote proliferation and invasion (Bourgot *et al.*, 2020). This increased ECM rigidity could explain the increased adhesiveness of the suspended neurospheres upon YAP1/WWTR1 overexpression.

Our findings are in accordance with prior work in SCLC, which has shown that cell lines fall into suspension and adherent subtypes corresponding with a NE and non-NE transcriptional profile, respectively (Horie *et al.*, 2016). A more detailed classification scheme, based on transcription factor expression, has been proposed for SCLC. NE SCLC cells can be specified by expression of the NE-associated transcription factors ASCL1 or NEUROD1, while non-NE cells are specified by expression of the chemosensory-associated transcription factor POU2F3 or YAP1 (Rudin *et al.*, 2019). Similar to our scRNA-seq findings in MCC (Ireland *et al.*, 2020) also found evidence for subpopulations of ASCL1-lo, YAP1-hi cells within otherwise ASCL1-hi SCLC tumors. Other reports have suggested that non-NE subpopulations such as these may be important for promoting metastasis (Kwon *et al.*, 2015) and drug resistance (Nguyen and Yi, 2019). In line with these ideas we observed a positive, albeit insignificant, trend of recurrent disease in patients with tumor biopsies classified as NE-lo.

Prior work has shown that YAP1 or WWTR1 expression is detrimental to the viability of NE-hi cancer cell lines (Nishikawa *et al.*, 2011; Pearson *et al.*, 2021). We observed that expression of either YAP1 or WWTR1 led to decreased proliferation of NE-hi MCCP cells in a TEAD-dependent manner.

Transcriptional profiling indicated that YAP1/WWTR1 expression led to general downregulation of cell-cycle-associated genes and MCPyV LT. In accordance with the role of MCPyV LT in inhibiting the activity of RB1 to prevent G1/S arrest (Hesbacher *et al.*, 2016), E2F target genes were downregulated and NE-hi MCCP cells dropped out of S-phase into G1 upon induction of YAP1 or WWTR1. We noted that YAP1 expression tended to have a more severe growth-suppressive phenotype than WWTR1 and was more effective at TEAD-dependently inducing gene expression. We speculate that this may reflect why some MCC tumors and PDCLs expressed WWTR1 and not YAP1, since WWTR1 may be less antagonistic than YAP1 to the NE transcriptional program.

Altogether these findings suggest that MCCP may, in particular, tend towards decreased levels of YAP1/WWTR1 due to the requirement of MCPyV T antigen expression. Indeed we found that the NMF group 2 were enriched for MCCP (77%, 20/26), while NMF group 1 were split (56% MCCP, 10/18). We speculate that the preference of MCPyV T antigen expression in the NE-hi state address the cell-of-origin of MCC. Given the ability of YAP1 and WWTR1 to repress MCPyV T antigen expression, we propose that only cells with low or absent expression of YAP1/WWTR1 will support MCCP oncogenesis.

Despite our work demonstrating that YAP1 overexpression transcriptionally suppresses MCPyV T antigen levels, prior work has shown that MCPyV ST overexpression can enhance YAP1 transcriptional activity through ST-mediated inhibition of protein phosphatase 2A (PP2A) (Nguyen *et al.*, 2014). Though these effects may be valid, this study was performed in primary non-NE human foreskin fibroblast (BJ) and mammary epithelial (HMEC) cells both of which expressed high levels of YAP1 protein at baseline. As a result, the indirect interaction between ST and YAP1 in the context of a non-NE state and overexpression may differ from that on the clonally integrated MCPyV genome in NE-hi, YAP1-silenced MCC.

Several questions regarding YAP1- and WWTR1-mediated MCPyV T antigen suppression remain. YAP1/TEAD and WWTR1/TEAD have previously reported transcriptional repressive activity in complex with the NuRD complex (Beyer *et al.*, 2013; Kim *et al.*, 2015). In addition, the related SV40 polyomavirus retains an TEAD-binding motif within the NCCR of its genome (Davidson *et al.*, 1988; Lin *et al.*, 2017). However, we did not observe a TEAD binding sequence in the MCPyV NCCR, suggesting repression may be an indirect effect. Furthermore, it is unclear why TEAD-binding defective YAP1 and

WWTR1 raise MCPyV LT levels. Depending on interacting partners, YAP1 and WWTR1 can have both transcriptional repressive and activating functions. It is possible that loss of TEAD-binding could promote transcriptionally activating YAP1 or WWTR1 binding partners that result in increased MCPyV LT levels.

This study has provided insight into previously unrecognized subgroups of MCC and drawn clear connections to other NE cancers. We have provided a model by which classic and variant MCC cell lines can be classified according to NE marker and YAP1/WWTR1 expression, and we suggest the importance of including variant MCC cell lines in future analyses. We have also provided molecular insight into the consequences of YAP1/WWTR1 expression in NE-hi MCCP cells. Our findings support the previously proposed idea that reciprocal expression of YAP1/WWTR1 and NE markers is a pan-NE cancer phenomenon, and we suggest further work be done to verify these data in other NE cancer types, including NE prostate cancer and medullary thyroid cancer.

Methods

Tissue Culture

Established MCC cell lines were cultured in RPMI-1640 with glutamine plus 10% FBS and 1% GlutaMAX. 293T cells were cultured in DMEM with glutamine and sodium pyruvate plus 10% FBS and 1% GlutaMAX. All cells tested negative for mycoplasma via PCR.

PDCLs were cultured in non-TC-treated flasks in Neurocult NS-A medium plus 10% NS-A proliferation supplement, 20 ng/mL human recombinant FGF, 20 ng/mL human recombinant EGF, 0.0002% heparin, and 1% penicillin/streptomycin (NS-A complete).

For isolation of single cells for scRNA-seq and establishment of PDCLs, patient-derived tumor biopsies were minced into approximately 1 mm segments and incubated for several hours at 37°C in dissociation media (NS-A complete with 2 mg/mL each of collagenase-IV and hyaluronidase) with regular mixing. The tumor suspension was passed through a 100 µm filter to remove large debris and isolate single cells. For PDCL establishment, cells were plated and cultured as above. For scRNA-seq, cells were plated overnight and submitted for library preparation the next morning. For scRNA-seq of MCC516, suspension and adherent cells were treated with Accutase and passed through a 70 µm filter to isolate single cells immediately prior to submission for library preparation.

Single-Cell RNA-seq

Sequencing libraries of single cell preparations from tumor biopsies or MCC516s/a were generated with the 10x Genomics Chromium Next GEM Single Cell 5' Kit v2. cDNA libraries were sequenced on the Illumina NovaSeq S4 platform to an average depth of 20,000 reads per cell. Cell Ranger was used to map reads to a hybrid of the Hg19 and MCPyV (R17b) genomes. Raw counts were input into Seurat and high-quality cells were identified as having <10% mitochondrial reads, >500 total UMIs, and >0.8 log₁₀ genes per UMI. The resulting 38,077 (tumor biopsies) or 9,150 cells (MCC516s/a) were analyzed in Seurat.

Bulk Tumor RNA-seq

Patient-derived tumor biopsies were either fresh-frozen and stored in liquid nitrogen or immediately treated with RNeasy Lysis Buffer (Qiagen) and stored at -80°C. Each RNeasy-treated sample was homogenized with the Qiagen TissueRuptor II and RNA was isolated with the RNeasy Spin Kit. The NEBNext Ultra II RNA Library Prep Kit for Illumina was used to prepare sequencing libraries. Paired-end sequencing (PE150) was performed on the NovaSeq 6000 system. Sequences were mapped and quantified to the decoy-aware, concatenated transcriptome of GRCh38.p13 (Ensembl v102) and MCPyV (R17b) using Salmon (Patro et al., 2017). Gene-level counts were generated via TxImport (Soneson et al., 2015) and normalized counts were generated via DESeq2 (Love et al., 2014). Samples were considered virus-positive if the number of normalized MCPyV LT counts was >100 and ST was >10.

Generation of Plasmid Constructs and YAP1 and WWTR1 Expressing Cell Lines

PLIX_402 (a gift from David Root, Addgene #41394) was used to generate inducible expression constructs. Constructs included YAP1 isoform 1-2 gamma (from Addgene #124145, a gift from Megan Finch-Edmondson and Marius Sudol), YAP1 1-2 gamma S94A, YAP1 1-2 gamma 5SA (S61/109/127/164/397A), YAP1 1-2 gamma 6SA (S61/94/109/127/164/397A), WWTR1 (from Addgene #82253, a gift from Jesse Boehm, William Hahn, and David Root), WWTR1 F52A, WWTR1 4SA (S66/89/117/311), WWTR1 4SA/F52A, or eGFP. PLenti CMV Blast DEST (a gift from Eric Campeau and Paul Kaufman, Addgene #17451) was used to generate constructs which contained MCPyV L21 truncated LT (Accession KC426955) or MCPyV L21 truncated LT E216K. 293T cells were transfected using PEI with pLIX_402 or pLenti constructs, psPAX2, and pMD2.G (gifts from Didier Trono, Addgene #12260 and #12259). Cells were transduced with lentivirus-containing supernatants by spinfection at 931g for 1 hour (WaGa) or 2 hours (MKL-1) at room temperature with 2 µg/mL polybrene. After spinfection 1 volume of complete RPMI was added. Cells were selected after 24 hours with 1 µg/mL puromycin for 3 days (WaGa) or 4 days (MKL-1) or with 20 µg/mL blasticidin for 6 days.

MKL-1 YAP1/WWTR1 RNA-seq

YAP1/WWTR1 expressing MKL-1 cells were treated with 2 µg/mL doxycycline for 3 days and RNA was extracted with TRIzol/chloroform. Library prep and sequencing were performed by Novogene.

Samples were sequenced on a Novaseq 6000 to obtain paired-end 150 bp sequences. Sequences were mapped and quantified as above. Differential expression analysis was performed via DESeq2 with comparisons of each condition to GFP. To obtain corrected target genes, lists of up and downregulated differentially expressed genes versus GFP were compared between YAP1 and YAP1 S94A or WWTR1 and WWTR1 F52A and overlaps were removed from the YAP1 and WWTR1 lists.

Viability Assays

YAP1/WWTR1 expressing MKL-1 cells were treated with 2 µg/mL doxycycline for 6 days with complete refreshment on day 3. At each timepoint, cells were treated with Accutase to disrupt clumps and suspensions were either plated in 96-well plates for a CellTiterGlo viability assay or lysed for SDS-PAGE.

Cell-Cycle Analysis

MKL-1 cells with inducible YAP1/WWTR1 were treated with 2 µg/mL doxycycline for 6 days with refreshment on day 3. On the final day cells were labeled with 10 µM 5-ethynyl-2'-deoxyuridine (EdU) in a final concentration of 0.1% DMSO for 1 hour prior to harvest. Cells were dissociated into single cells with Accutase and 2 million per sample were fixed in in sub-zero (-20°C) 70% ethanol. The remaining cells were lysed. Alexa Fluor-647 azide was conjugated onto incorporated EdU via copper I-catalyzed click chemistry. Cells were stained with DAPI to measure total DNA content. Stained cells were passed through a 70 µm filter and flow cytometry was used for cell cycle analysis. Doublets were removed via DAPI height verses area discrimination. A minimum of 30,000 events were recorded per sample.

Immunohistochemistry

Immunohistochemistry was performed on the Leica Bond automated staining platform using the Leica Biosystems Refine Detection Kit. Antibodies and dilutions used for IHC include: 1:200 rabbit anti-ATOH1 (Proteintech, #21215-1-AP) and 1:100 mouse anti-YAP1 (Santa Cruz, #SC101199) each with citrate antigen retrieval; 1:100 rabbit anti-WWTR1 (Cell Signaling, #72804) and 1:50 mouse anti-KRT20 (DAKO, #72804) each with EDTA antigen retrieval.

Immunoblot

Cell pellets were lysed in RIPA buffer plus protease and phosphatase inhibitors. Lysates were run on a gradient SDS-PAGE gel, followed by transfer to PVDF and blocking in 5% milk. Blots were incubated at 4°C overnight in primary antibody and imaged with SuperSignal West Femto substrate. Primary antibodies and concentrations used in this study include: 1:1000 rabbit anti-YAP1 Cell Signaling #14074, 1:1500 rabbit anti-YAP1/WWTR1 Cell Signaling #8418, 1:1000 rabbit anti-LATS1 Cell Signaling #3477, 1:1000 rabbit anti-LATS2 Cell Signaling #5888, 1:1000 rabbit anti-MST1 Cell Signaling #3682, 1:1000 rabbit anti-MST2 Cell Signaling #3952, 1:1000 rabbit anti-pT35 MOB1 Cell Signaling #8699, 1:1000 rabbit anti-MOB1 Cell Signaling #13730, 1:1500 rabbit anti-TBP Cell Signaling #8515, 1:30,000 mouse anti-vinculin Sigma Aldrich #V9131, 1:1000 rabbit anti-ATOH1 Proteintech #21215-1-AP, 1:500 mouse anti-SOX2 R&D Systems #MAB2018, 1:1000 rabbit anti-CHGA Cell Signaling #60893, 1:1000 rabbit anti-synaptophysin Cell Signaling #5461, 1:500 mouse anti-KRT20 Thermo #MS-377-S0, 1:500 mouse Ab5 (5, 69), 1:1000 rabbit anti-GFP Cell Signaling #2956.

RT-qPCR

To determine baseline *YAP1/WWTR1* expression in cell lines, RNA was extracted with the Qiagen RNeasy kit. To measure expression changes in *YAP1/WWTR1* expressing MKL-1 cells, cells were treated with 2 µg/mL doxycycline for 3 days and RNA was extracted with TRIzol/chloroform. Reverse transcription was carried out with the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit. RT-qPCR was performed using Brilliant III Ultra-Fast Sybr Green QPCR Master Mix with 40 cycles of 2-step amplification with a 60°C T_m. Data was analyzed according by the $\Delta\Delta C_t$ method with normalization to 18S rRNA. Primers used in this study include: 18S rRNA (5'-AACCCGTTGAACCCCAT-3', 5'-CCATCCAATCGGTAGTAGCG-3'), MCPyV LT (5'-ATTGAGCTTCGGGAAGGCATAC-3', 5'-GCTCCCCTGGATGCATTGG-3'), MCPyV ST (5'-AGGTCCTGGGTGCATGCTT-3', 5'-ACACTCTCCCCACGTCAGAC-3').

Microscopy

Images were taken at 4x or 10x using SPOT5.2 software on a Nikon Eclipse TE300 inverted microscope with a Diagnostic Instruments Model #25.4 2 Mp Slider Camera.

Statistics

All statistical tests were performed using GraphPad Prism. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Specific statistical tests are outlined in the figure or table legends.

Data Availability Statement

MKL-1 YAP1/WWTR1 transcriptional profiling data is available through NCBI GEO at accession number GSE189054 (reviewer key edyvouemnjuhiti). The bulk tumor and PDCL RNA-seq datasets are available in a recently accepted manuscript (151666-JCI-RG-RV-3). The patient-derived scRNA-seq datasets are available upon request.

Study Approval

This study was approved by the DFCI Institutional Review Board for use of human tumor samples (IRB protocol #09-156). Written informed consent was received prior to patient participation in the study.

Chapter 4: Discussion

MCC is a poorly understood NE malignancy with limited treatment options for metastatic disease. In this work we identified novel, therapeutically-relevant stratifications of MCC populations based upon *TP53*-status and expression of NE genes. Regarding the former, we explored the potential of IMPDH as a therapeutic target in MCC. We found that IMPDH inhibition decreased cellular guanylate nucleotide pools and induced p53-dependent cytotoxicity in MCC. We identified nascent DNA but not RNA synthesis to be rapidly ablated following IMPDH inhibitor treatment, supporting a model where deoxyguanylate nucleotides are initially limiting. Concordant with these results, we identified RS as the dominant consequence of IMPDH inhibition and subsequent p53 activation to depend on the induction of DDK activity. Building on this work, we utilized an ATR inhibitor to enhance the effectiveness of IMPDH inhibition-induced RS to sensitize *TP53*-mutant MCC to IMPDH inhibitors through the induction of RC. This work identifies *TP53*-status as a key therapeutic stratification marker in MCC and suggests the potential of IMPDH and ATR inhibitor-based treatment strategies. In the latter work, we evaluated the expressional heterogeneity of MCC through transcriptomic and proteomic analyses of patient-derived tumor biopsies and cell lines. Through these high-throughput assessments, we identified Hippo pathway transcription factors YAP1 and WWTR1 to be highly negatively correlated with the expression of a NE transcriptional program in bulk and single-cell populations, at the RNA and protein levels, in patient tissues and derived cell lines. This led us to reclassify MCC broadly into NE-hi or NE-lo subtypes, based upon expression of NE genes or YAP1 and WWTR1, respectively. Mechanistically, we found that overexpression of YAP1 or WWTR1 was broadly anti-proliferative in NE-hi MCCP cell lines through the suppression of MCPyV TAg expression and reinstatement of RB1-dependent cell-cycle arrest. This work has suggested a previously unrecognized and pervasive presence of non-NE cells within MCC tumors and identifies YAP1 and WWTR1 as novel MCPyV TAg restriction factors. Here we explore an unexpected relationship between the cell-of-origin for MCCP and MCPyV infection and the expression of YAP1 and WWTR1. In addition, we expand our findings to comment on the broader potential of *TP53*- and NE-status as therapeutic stratification markers in metastatic MCC.

A potential mechanism for YAP1- and WWTR1-mediated repression of MCPyV gene expression and implications for MCPyV replication dynamics and MCCC cell-of-origin.

A long-standing question in the field of MCC is the cell-of-origin for MCPyV-mediated MCC, or MCCC. Normal Merkel cells have been proposed to be the cell-of-origin due to the similarity in NE gene expression observed in these cells and MCCC, including the Merkel cell-unique marker KRT20 (Tang and Toker, 1978; Toker, 1972). However, not all markers are shared between MCCC and Merkel cells (Tilling and Moll, 2012) and, in addition, Merkel cells are highly differentiated and non-proliferative (Moll et al., 1996). As a result, other candidates for the cell-of-origin for MCCC have been suggested. Indeed, B-cells have also been implicated due to expression of paired box gene 5 (PAX5), a marker of pro-/pre- or pre-B cells, in clinical tissues of MCCC (Zur Hausen et al., 2013). However, PAX5 is also observed to be expressed in non-hematopoietic cells such as NE cancers or normal neuronal tissues (Torlakovic et al., 2006), indicating this marker is not exclusive to B cells. Epidermal and dermal stem cells in addition to other skin-derived precursors have been suggested (Tilling and Moll, 2012), although none have been conclusively linked to MCPyV infection or development of MCCC. Recently, the generation of an MCC-like tumor was observed upon co-expression of MCPyV TAg and ATOH1 in addition to the knockout of p53 in a keratin 5 (KRT5) promoter-driven mouse model (Verhaegen et al., 2022). Interestingly, normal Merkel cells arise from keratin 14 (KRT14)/KRT5 dual-positive basal epidermal progenitors through ATOH1-dependent transcriptional reprogramming (Morrison et al., 2009). However, whether these KRT14/KRT5 dual-positive basal epidermal cells or their descendants are the cell-of-origin for MCCC and MCPyV infection in physiological circumstances remains unknown.

A few bare-minimum criteria exist for a potential cell-of-origin for MCCC: (1) they must support entry of MCPyV, (2) they must be permissive for expression of MCPyV genes, and (3) they must contain integrated MCPyV DNA. However, not through lack of effort, a specific cell type able to effectively host MCPyV infection and subsequent MCCC formation has not been identified.

To study MCPyV entry and replication kinetics several systems to generate MCPyV genomic DNA and virions have been described. MCPyV genomic DNA can be propagated by transfection of 293 cells expressing SV40 LT (293TT) with the genome of a clinical isolate (Neumann et al., 2011) or a consensus genomic sequence from compiled clinical isolates (a “synthetic” genome) (Schowalter et al.,

2011) that are subcloned into a carrier plasmid which contains an SV40 origin of replication. In this way, the SV40 LT can recognize the SV40 origin of replication and permit efficient replication of MCPyV DNA within the recombinant vector, which alone does not replicate or express well (Neumann *et al.*, 2011; Schowalter *et al.*, 2011). Liberation of the replicated MCPyV genome from the carrier plasmid and subsequent re-ligation allows for transfection of MCPyV genomes to study permissive infection. “Native” virus can also be produced by transfecting these re-ligated MCPyV genomes into 293TT cells concurrently with expression plasmids for MCPyV ST and LT (Schowalter *et al.*, 2011). This method allows for encapsulation and recovery of MCPyV virions, though this process requires large quantities of materials and is similarly inefficient. Finally, a recombinant MCPyV reporter pseudovirus can be generated through transfection of VP1 and VP2 expression plasmids concurrent with a reporter plasmid under the control of the cytomegalovirus immediate early promoter, or other strong promoter (Schowalter *et al.*, 2011). VP1 and VP2 can self-assemble into virus-like particles and encase MCPyV genome-sized DNA fragments through a direct interaction between VP1 and DNA (Gillock *et al.*, 1997). In this context, MCPyV entry can be monitored through expression of the reporter gene after inoculation of cells with the MCPyV reporter pseudovirus.

Entry of MCPyV virions is thought to be mediated through an initial attachment between the icosahedral capsid composed of VP1/VP2 and heparan sulfate (HS)-coated proteoglycans (Schowalter *et al.*, 2011). This association is followed by an additional interaction between VP1 and sialic acid (SA) which is essential for entry (Neu *et al.*, 2012). HS and SA are ubiquitous sugars moieties which are often present on the terminal ends of glycoproteins that reside on the plasma membrane or extracellular matrix (Mathez and Cagno, 2021). These residues are implicated in the attachment and entry of numerous viruses, from herpes simplex viruses (HSV) to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and do not propend specificity to any cellular lineage (Mathez and Cagno, 2021). In addition, no specific protein receptor has been identified for MCPyV which could indicate preferential infection of cell types (Liu and You, 2020). Indeed, MCPyV reporter pseudovirus poorly enters a wide variety of cell types from the NCI-60 panel of 60 human cancer cell lines which are known to express HS- and SA-coated proteins (Schowalter *et al.*, 2012). Even established MCCC cell lines are refractive to entry of MCPyV

reporter pseudovirus (Schowalter *et al.*, 2012), although it was not shown if these cells were permissive for MCPyV genome replication.

Concurrent with the lack of clarity regarding MCPyV entry kinetics, no physiologically-relevant *in vitro* model exists that efficiently permits MCPyV replication. Transfection of synthetic MCPyV genomes into a variety of transformed cell lines is poorly efficient for genome replication and gene expression with one study showing 3 of 21 transfected cell lines able to replicate any amount of DNA or express any MCPyV protein (Neumann *et al.*, 2011). Of these 3 cell lines, expression of LT was lost over several days while VP1 expression was minimal throughout. In addition, no infectious virus was recovered from these cells although irregularly-shaped virus-like particles were observed following infection in extremely low abundance as compared to similarly transfected, and replication-competent, SV40 genomes (>10,000-fold fewer virions). Lack of infectious virus production despite low levels of genomic replication and gene expression was also observed by another group in a similar assay but using a different synthetic MCPyV genome (Feng *et al.*, 2011). As such, although expression of MCPyV proteins in addition to genome replication can be observed upon transfection, these are poorly efficient processes in the cell lines currently assessed.

Recently it has been shown that human dermal fibroblasts could inefficiently support the entire MCPyV lifecycle in non-physiological contexts (Liu *et al.*, 2016). To identify the natural host cell of MCPyV infection, Liu *et al* applied both “native” and reporter MCPyV virions to the total cell population of primary human foreskins. The authors noted that cells expressing cytokeratin 14 (KRT14), a lineage marker of proliferative epidermal basal cells, could support minimal entry of reporter pseudovirus but not replication of “native” MCPyV virions. In addition, KRT20-positive Merkel cells did not efficiently support either entry or replication, though the latter could be due to lack of virion entry. The only cell type which could support MCPyV entry and replication were human dermal fibroblasts, albeit with extremely poor efficiency (< 0.5% LT-positive cells). This infection could be modestly improved by switching between human-derived and bovine-derived growth factors concurrent with treatment with a glycogen synthase kinase 3 (GSK3) inhibitor (CHIR99021) during infection. In addition to these alterations, a multiplicity of infection (MOI) of 100,000 “native” virions per cell was required to observe MCPyV LT expression in even ~5% of human dermal fibroblast cells *in vitro*. Transfection of a clinical isolate MCPyV genome in identical conditions

allowed the production of virions which were capable of infecting other human dermal fibroblasts again under these conditions. As a result, addition of exogenous factors can predispose human dermal fibroblasts to produce MCPyV virions although physiologically-relevant conditions remain unclear.

Concordant with this work, high MOI infection with “native” MCPyV virions resulted in poorly permissive expression of MCPyV genes in numerous cellular contexts (Liu *et al.*, 2018; Schowalter *et al.*, 2012; Tsang *et al.*, 2014). Even in individual transformed epidermal cells which were positively infected with “native” MCPyV virions, only ~1% of these displayed co-localization of LT with the MCPyV genome, an indicator of active DNA replication (Tsang *et al.*, 2014). Indeed, decreases in MCPyV genome copies over time were observed in several cell lines from a variety of lineages (Schowalter *et al.*, 2012), indicating the possibility of host-dependent restriction mechanisms. Preemptive co-expression of both MCPyV and SV40 TAg supported an increase in MCPyV genomic copies although it was not assessed if infectious virions were generated (Schowalter *et al.*, 2012).

To summarize the current state of the literature: transfection or “native” infection with virions inefficiently induces expression of MCPyV genes and can support limited genomic replication in a minority of cell lines. Addition of high concentrations of non-physiological chemicals and growth factors are required to produce even small amounts of infectious MCPyV virions. As a result, it is currently unknown what host cell provides a physiologically-relevant and permissive environment for MCPyV replication or what restriction factors hinder replication within these non-permissive cells.

In Chapter 3, we identified Hippo pathway transcription factors YAP1 and WWTR1 as restriction factors for MCPyV TAg expression. Specifically, overexpression of YAP1 or WWTR1 suppressed MCCP proliferation (Figure 3.17A) through the downregulation of MCPyV LT RNA (Figure 3.17B, 3.17C) and protein levels (Figure 3.19B, 3.20A), leading to reinstatement of RB1-dependent cell-cycle dysregulation (Figure 3.19D). YAP1 and WWTR1 are broadly expressed in most tissues and cell lines (Harvey *et al.*, 2013; Rognoni and Walko, 2019), including NE-lo MCC cell lines but are not expressed in NE-hi MCC cell lines (Figure 3.3, Figure 3.8). Similar to these observations, YAP1 expression is also present in a non-NE SCLC subtype (Pearsall *et al.*, 2020; Rudin *et al.*, 2019). Concordantly, we also found that the expression of NE genes and YAP1- or WWTR1-associated gene sets are negatively correlated in MCC (Figure 3.3), as is also observed in SCLC (Pearsall *et al.*, 2020; Rudin *et al.*, 2019). Furthermore, the NE-hi NMF

subgroup is enriched for MCCR tumor samples (Figure 3.1), and all derived MCCR cell lines exclusively express a NE transcriptional program and not YAP1 or WWTR1 (Figure 3.3, Figure 3.8). Taken together, these results suggest a preference for MCPyV gene expression in cells retaining a NE transcriptional program. Furthermore, these results indicate that induction of YAP1 or WWTR1 can suppress MCPyV gene expression through a yet unclear mechanism.

A direct mechanism of YAP1- or WWTR1-mediated control of MCPyV gene expression may be through interaction with the MCPyV NCCR. Indeed, YAP1 and WWTR1 can be transcriptional activators or repressors (Kim *et al.*, 2015; Kim *et al.*, 2018), though these activities are context dependent. Additionally, although YAP1 and WWTR1 are transcription factors they lack intrinsic DNA-binding capabilities and thus must interact with the DNA-binding TEAD1-4 proteins for chromatin localization and subsequent gene regulation (Kim *et al.*, 2018). The related polyomavirus SV40 contains a consensus TEAD-binding motif in its NCCR (Davidson *et al.*, 1988; Lin *et al.*, 2017) which is responsible for promoting TAg expression. As such, we speculated that the MCPyV NCCR may have a similar TEAD-binding motif and that YAP1- and WWTR1-mediated TAg suppression may be through direct interaction with the MCPyV NCCR. However, assessment of the MCPyV NCCR did not reveal a consensus TEAD motif as was observed for the SV40 NCCR (Figure 3.20). As a result, the effects of YAP1 and WWTR1 on MCPyV TAg expression are likely indirect.

Recently at the 2nd International Merkel Cell Carcinoma Symposium, it was suggested by Isaac Brownell that expression of MCPyV TAg is controlled by cooperation of the LIM homeobox 3 (LHX3) and ISL1 transcription factors (Brownell, personal communication, April 2022). LHX3 and ISL1 are LIM homeobox domain-containing proteins which function to bind and control the expression of genes critical for neuron differentiation in conjunction with LDB1. LDB1 is pleiotropically expressed, has no DNA binding activity, and functions as a scaffold to associate with numerous other LIM-domain-containing proteins to drive diverse developmental processes (Matthews and Visvader, 2003). In the context of LDB1, LHX3 and ISL1, three separate complexes have been shown to form via LIM-domain interactions: LDB1-LHX3, LDB1-ISL1, and LDB1-LHX3-ISL1. It has been shown that LHX3-LDB1 complexes drive interneuron differentiation, LHX3-ISL1-LDB1 complexes drive motor neuron differentiation (Kadmas and Beckerle, 2004), while ISL1-LDB1 complexes are critical for cardiac lineage differentiation (Caputo *et al.*,

2015) and islet β -cell development (Hunter et al., 2013). It has been speculated that the interaction between ISL1 and LHX3 modulates DNA binding specificity and that ISL1 alone binds to DNA poorly (Smith et al., 2021). We (Chapter 3) and others (Agaimy *et al.*, 2013; Gravemeyer *et al.*, 2021) have shown that MCC widely expresses ISL1 and upon reassessment of our bulk tumor RNA-seq, PDCL RNA-seq, and established cell line RNA-seq we found similar broad expression of LHX3, especially in the MCCP subtypes or in samples with higher expression of NE genes (Figure 4.1). Interestingly, normal Merkel cells also highly express both ISL1 and LHX3, indicating the potential for ISL1-LHX3-containing complexes to naturally form in these cells (Haeberle et al., 2004). In addition, LHX3 but not ISL1 is essential for MCCP survival via CRISPR screening (Figure 4.2) (Cheng *et al.*, 2017), indicating that LHX3 is a critical transcription factor in MCCP and possibly in Merkel cells. Finally, assessment of the NCCR of MCPyV revealed an LHX3 consensus motif (5'-TAATTA-3'; Figure 4.3), which could be responsible for driving MCPyV TAg expression as speculated by Brownell. However, an association between YAP1- or WWTR1-mediated MCPyV TAg suppression and LHX3- and ISL1-containing complexes was yet unclear.

Reassessment of YAP1 or WWTR1 overexpression in NE-hi MCCP cells revealed the upregulation of another LIM-domain containing protein, ajuba LIM protein (AJUBA; Figure 4.4). AJUBA is abundantly expressed in adult skin, and interacts via LIM-domain interactions to mediate development of epidermal cells (Schleicher and Schramek, 2021), among other functions. Interestingly, AJUBA was found to directly bind to ISL1 via LIM:LIM domain interactions and suppress ISL1 transcriptional activity in cardiac cells (Witzel et al., 2012). Interaction between LHX3 and AJUBA has not been reported, though could occur through a similar mechanism. We also observed a positive correlation of AJUBA expression with YAP1- or WWTR1-associated genes sets, and a negative correlation with NE gene expression, including LHX3 and ISL1 in our RNA-seq datasets (Figure 4.1). In other work, AJUBA has been shown to be induced by YAP1 *in vitro* and *in vivo* in multiple cell types (Horie *et al.*, 2016; Lange et al., 2015). Highly interesting, knockdown of AJUBA in an adherent non-NE SCLC cell line induced increased circularity of these cells (Horie *et al.*, 2016), reminiscent of neurospheres observed in other NE SCLC cell lines. As a result, it is possible that YAP1 or WWTR1 expression induces AJUBA to directly suppress ISL1- and LHX3-mediated MCPyV TAg expression and that YAP1/WWTR1/AJUBA-mediated activity may be restricted from NE cells.

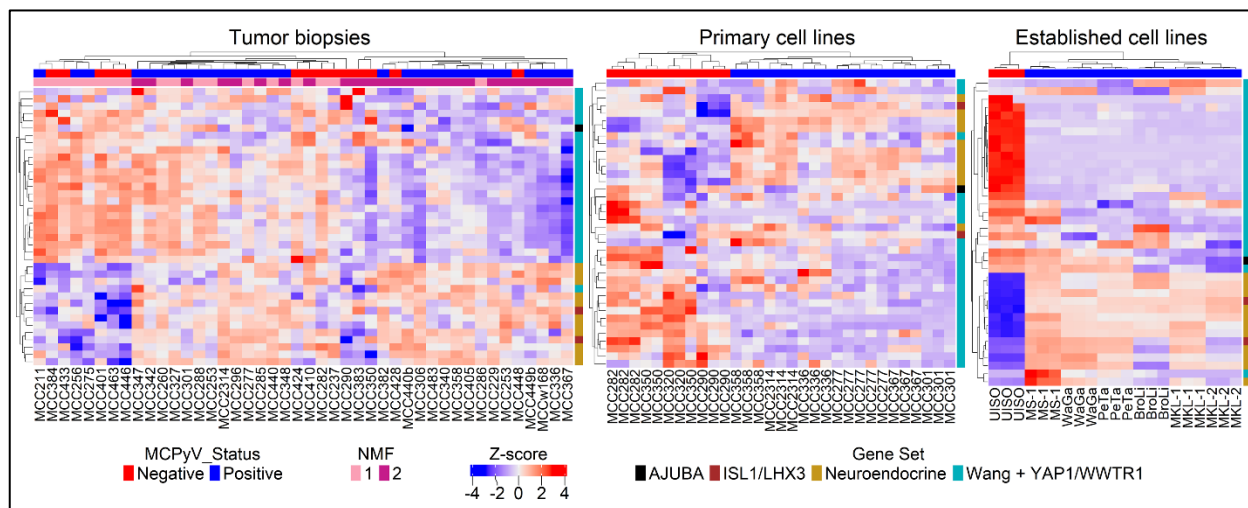


Figure 4.1 NE-hi MCC samples are correlated with expression of LHX3 and ISL1, and negatively correlated with AJUBA. Reanalysis of RNA-seq datasets from patient-derived tumor biopsies, patient-derived cell lines, and established cells lines as in Figure 3.3C, with inclusion/highlighting of LHX3, ISL1, and AJUBA.

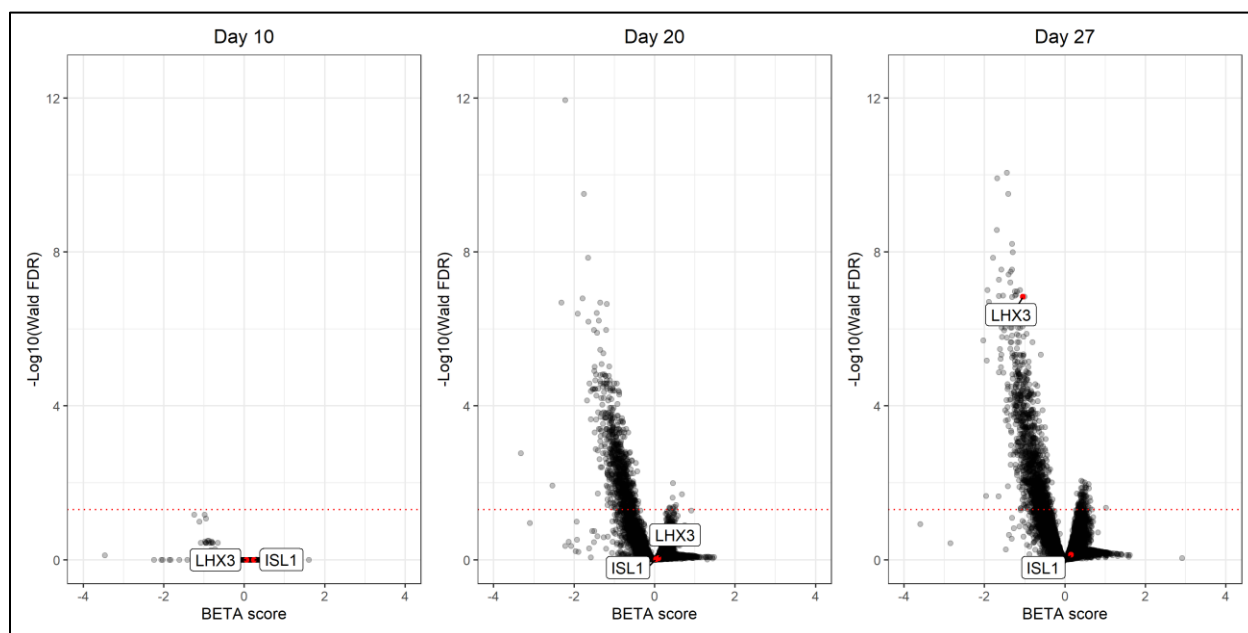


Figure 4.2 LHX3 is essential for survival of an NE-hi MCCP cell line. Reanalysis of genome-wide CRISPR knockout screen from Cheng et al and Park et al, as in Figure 1.2, with inclusion/highlighting of LHX3 and ISL1. Red dotted line indicates Wald FDR < 0.05. BETA score roughly indicates Log₂-fold change in guides from study initiation to specific time point harvested.

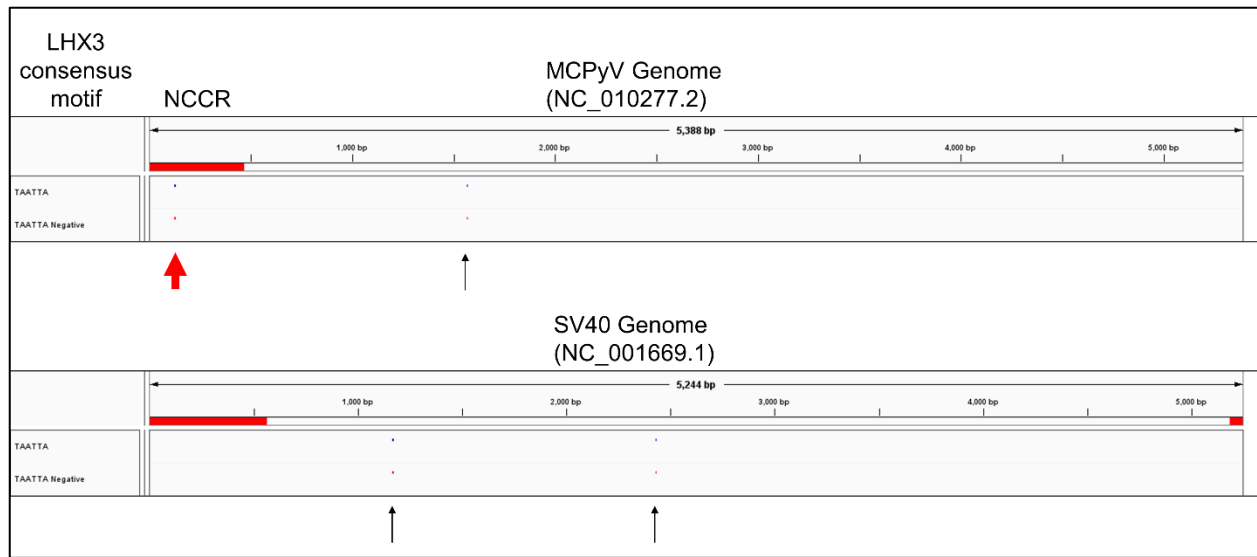


Figure 4.3 An LHX3 consensus binding motif is present in the NCCR of the MCPyV genome. Reanalysis of MCPyV and SV40 genomes as in Figure 3.20, but for LHX3 consensus motif (5'-TAATTA-3'). Red line indicates the NCCR. Red arrow indicates consensus motif present within the NCCR, black indicates consensus motif outside the NCCR.

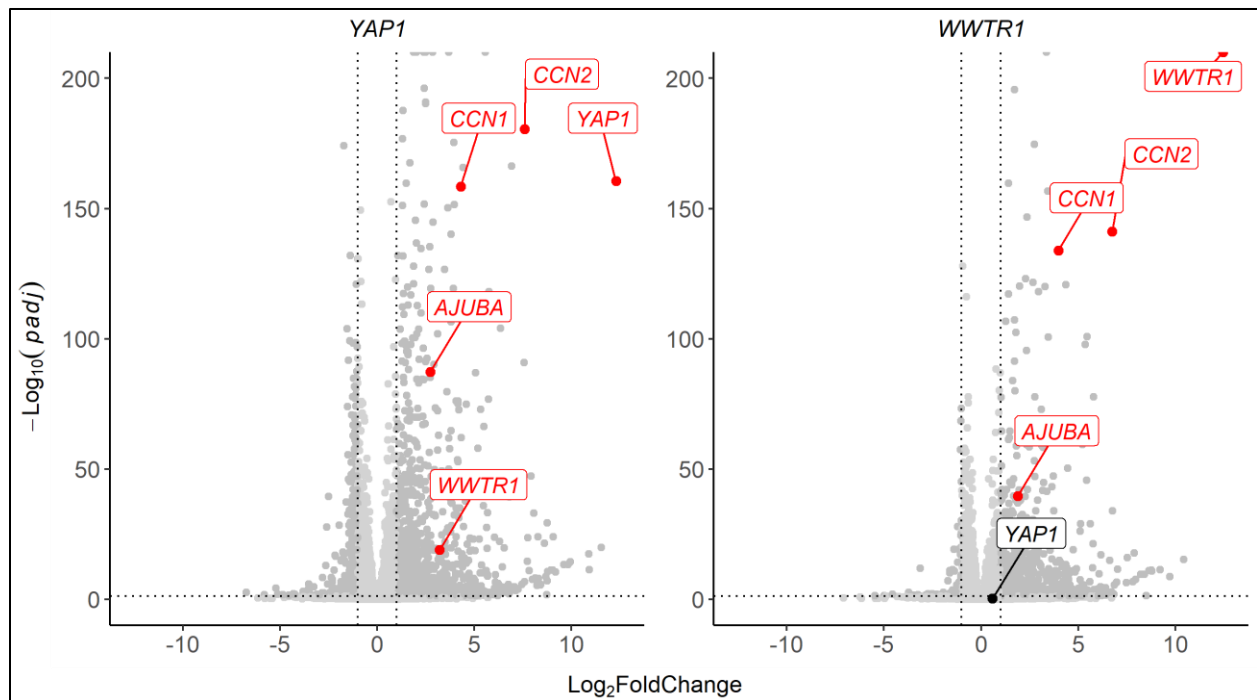


Figure 4.4 YAP1 or WWTR1 overexpression induces AJUBA in a NE-hi MCCP cell line. Reanalysis of YAP1 and WWTR1 overexpression in NE-hi MCCP cells as in Figure 3.17B, with AJUBA additionally highlighted. Red indicates $> 1 \log_2$ -fold change and $padj < 0.05$; also shown by dotted black lines.

As a gestalt, our work (Chapter 3) synthesizes other findings in the field to suggest a cohesive and dynamic model for the cell-of-origin for MCCP. Numerous reports have shown that MCPyV virions poorly enter and replicate in a variety of transformed and normal cell lines including those present within the skin (Liu *et al.*, 2018; Liu *et al.*, 2016; Schowalter *et al.*, 2012; Tsang *et al.*, 2014). Human dermal fibroblasts are capable of supporting MCPyV entry and replication, but only with non-physiological stimuli (Liu *et al.*, 2016), implying the need for a modified cellular state to support MCPyV replication. Specifically, this enhanced MCPyV infection required switching between human and bovine growth factors in addition to inhibition of GSK3 in dermal fibroblasts (Liu *et al.*, 2016). Liu *et al.* also noted that KRT14/KRT5 dual-positive basal epidermal progenitor cells support entry of MCPyV reporter pseudovirus but not native MCPyV virion infection (Liu *et al.*, 2016). KRT14/KRT5 dual-positive basal epidermal progenitor cells are known to have high levels of YAP1 activity that support a stem-like state, which is decreased over time in derivative cells as they terminally differentiate (Rognoni and Walko, 2019). Interestingly, other work has shown that inhibition of GSK3 decreases protein levels and activity of YAP1 (Santoro *et al.*, 2020), indicating that the GSK3 inhibition of dermal fibroblasts (Liu *et al.*, 2016) may disrupt YAP1 activity to permit MCPyV replication. Agreeing with this idea, our work has identified YAP1 and WWTR1 as suppressors of MCPyV TAg expression (Chapter 3). Independently, Isaac Brownell recently suggested that MCPyV TAg expression depends on neuronally-associated ISL1/LHX3 transcription factors (Brownell, personal communication, April 2022) which we (Chapter 3, Figure 4.1, 4.2, 4.3) and others (Agaimy *et al.*, 2013; Gravemeyer *et al.*, 2021; Haeberle *et al.*, 2004) have shown to be highly expressed in normal Merkel cells and MCCP. Tying these lines of reasoning, overexpression of YAP1 or WWTR1 induces expression of AJUBA (Figure 4.4), previously shown to inhibit the transcriptional activity of ISL1 (Witzel *et al.*, 2012) and potentially LHX3. In agreement with this idea, we have shown a negative correlation between the NE transcriptional program in MCC (including ISL1 and LHX3) with expression of YAP1, WWTR1, and AJUBA (Chapter 3, Figure 4.1). Strikingly, normal Merkel cells are derived from a KRT14/KRT5 dual-positive basal epidermal lineage (Morrison *et al.*, 2009) and undergo differentiation to express ISL1/LHX3 and suppress YAP1/WWTR1/AJUBA (Haeberle *et al.*, 2004). It is therefore possible that expression of MCPyV genes can occur in normal Merkel cells, and that this expression could drive the differentiated Merkel cells into a proliferative state. Indeed, MCPyV ST, in

the context of the SLAP transcriptional co-activator complex, was shown to induce expression of a lysine demethylase 1A (KDM1A or LSD1)-driven histone demethylase complex (Park *et al.*, 2020). This repressive complex was found to bind the transcriptional start sites of genes also bound by ATOH1 (Park *et al.*, 2020), the driving transcription factor essential for normal Merkel cell development (Morrison *et al.*, 2009; Ostrowski *et al.*, 2015). It may be the case that MCPyV ST acts to deprogram ATOH1-driven terminal differentiation programs through KDM1A-driven repression of ATOH1-dependent gene expression. Furthermore, MCPyV can suppress the activity of key tumor suppressor RB1 and p53. Specifically, LT can directly interact with and suppress RB1-mediated G1/S arrest while ST (Cheng *et al.*, 2013), still in the context of SLAP complex, can transactivate MDM2 to suppress p53 levels (Park *et al.*, 2019). Combined, these MCPyV TAg-driven perturbations may contribute to proliferation and dedifferentiation of Merkel cells to promote MCPyV replication and possibly the formation of MCCP. Taken together, our work suggests a “two-cell” model by which KRT14/KRT5 dual-positive basal epidermal progenitor cells are entered by MCPyV virions, albeit inefficiently. These MCPyV-infected cells can subsequently undergo differentiation into Merkel cells who, with expression of ISL1/LHX3 and repression of YAP1/WWTR1/AJUBA, can support MCPyV gene expression and transformation into MCCP (Figure 4.5).

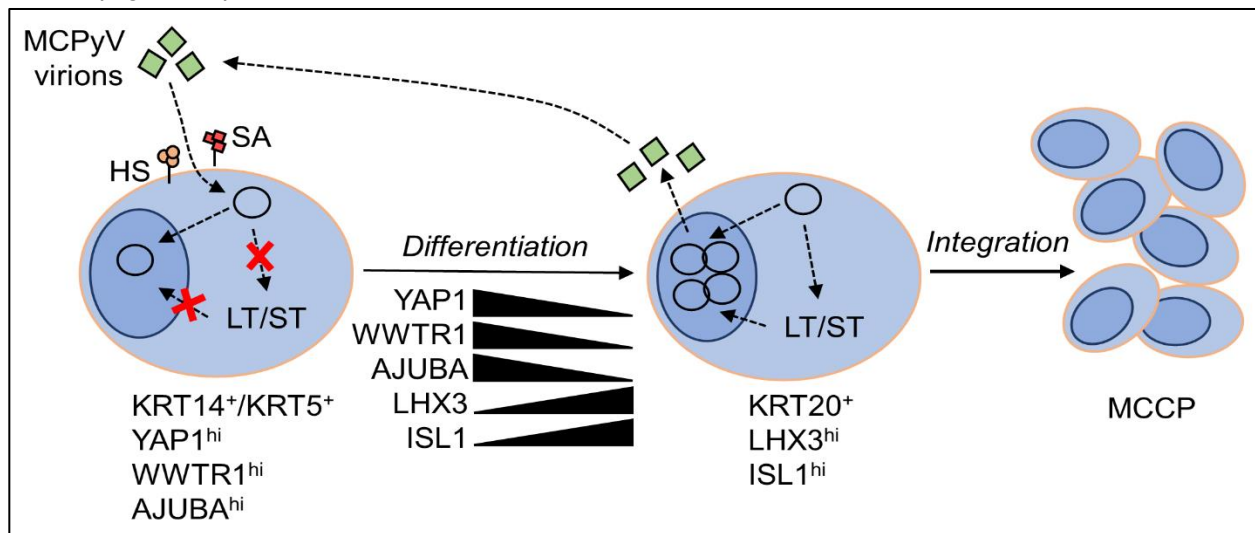


Figure 4.5 Mechanistic diagram of “two-cell” model of MCPyV cell-of-origin. MCPyV virions use heparin sulfate (HS) and sialic acid (SA) to enter KRT14/KRT5-positive basal epidermal progenitor cells, but gene expression and genome replication are restricted by high levels of YAP1/WWTR1/AJUBA. Differentiation into Merkel cells allows the upregulation of LHX3 and ISL1 concordant with YAP1/WWTR1/AJUBA downregulation. This transcriptional change promotes an environment conducive to robust MCPyV gene expression and virion production and, in some cases following integration of the MCPyV genome into the host genome, the formation of MCCP.

Our proposed “two-cell” model puts several observations regarding Merkel cells as the cell-of-origin of MCCP into perspective. Foremost, it is thought that Merkel cells are post-mitotic and non-proliferative and thus could not account for the millions of MCPyV virions consistently shed from healthy human skin (Schowalter et al., 2010). Our model suggests that since KRT14/KRT5 dual-positive basal epithelial cells are widely present in the human skin (Rognoni and Walko, 2019), they represent a large reservoir of cells that MCPyV is capable (Liu *et al.*, 2016), albeit poorly, of entering. Cellular tropism for the basal epithelium is also observed in the related oncogenic HPV family of viruses (Lei et al., 2020). Access to the basal epidermis for HPV infection is thought to occur through the accumulation of microlesions in the overlying skin (McMurray et al., 2001). As a result, it is possible that MCPyV virions enter KRT14/KRT5 dual-positive basal epidermal cells through a similar mechanism. Basal epidermal cells differentiate into numerous epidermal cell types including Merkel cells (Morrison *et al.*, 2009). As such, MCPyV may enter large populations of KRT14/KRT5 dual-positive epidermal basal cells and seed subsequently derived and differentiated cells with MCPyV genomic DNA. This process of differentiation could then support robust MCPyV gene expression in certain cellular contexts, as is observed in natural HPV infection (Lei *et al.*, 2020). Although in many cases these differentiated cells are unlikely to result in robust MCPyV replication, MCPyV DNA may be present in numerous cells within the epithelium, albeit likely at low copy number. The accumulation of MCPyV genomes in the epidermis through seeding by the basal epidermal layer could explain the seeming ubiquity of MCPyV DNA observed in swabs of patient skin samples, which rely on PCR-based amplification methods for detection (Foulongne et al., 2010). Furthermore, Merkel cells may efficiently support MCPyV replication through expression of ISL1/LHX3 to drive the generation and shedding of newly generated MCPyV virions, or in some rare instances, integrate to form MCCP. In conjunction, MCPyV TAg have several mechanisms to reverse differentiation and promote proliferation of Merkel cells (Cheng *et al.*, 2013; Park *et al.*, 2019; Park *et al.*, 2020), suggesting that their normally post-mitotic state could be overcome.

Another concern brought against a Merkel cell cell-of-origin is the observation that MCCP are often found in the dermal and not epidermal layer of skin (Hoefler et al., 1984) and therefore a dermal cell-of-origin is favored. Normal Merkel cells are found attached to the basal layer of epidermis, which divides the dermal and epidermal components of the skin via the basement membrane, to transduce

touch-stimuli through their innervated somatosensory fibers to the underlying tissue (Munde et al., 2013). Concordant with this, Merkel cells are also associated with hair follicles, which although surrounded by epidermal cells and the basement membrane, extend deep into the dermal layer (Rognoni and Walko, 2019). Epidermal cancers often invade through the basement membrane, which is considered an essential step for the development of metastasis (Chang and Chaudhuri, 2019). Invasive behavior is often linked to overexpression of matrix metalloproteinase (MMPs), which are important for degrading the dense sheet of extracellular matrix proteins, typically laminin and collagen IV, which are the dominant components of the basement membrane (Chang and Chaudhuri, 2019). Interestingly, MCPyV ST expression has been shown to activate MMP-9 to degrade collagen IV and promote invasion through a collagen IV-coated membrane (Nwogu et al., 2020). Indeed, high expression of MMPs is linked to enhanced metastatic activity (Fernandez-Figueras et al., 2007) and worse prognosis (Massi et al., 2003; Suomela et al., 2009) broadly in MCC. As such, due to the proximity of Merkel cells to the basement membrane, the propensity of epithelial cancers to invade through this barrier (Chang and Chaudhuri, 2019), and the ability of MCPyV ST to induce MMP-9-mediated invasion, it is feasible that the apparent dermal presentation of MCCP could be observed. It should also be noted that purely intraepidermal MCCP has also been observed (Harms *et al.*, 2018), further supporting the idea of an epidermal cell-of-origin, such as Merkel cells, for MCCP.

Finally, our model explains the poor permissivity for MCPyV replication in all cell lines currently assessed. According to our model, expression of YAP1, WWTR1, and AJUBA should suppress while LHX3 and ISL1 should support MCPyV replication. Assessment of microarray gene expression data from the NCI-60 cell lines (Ross et al., 2000; Scherf et al., 2000), which is composed of cancer cell lines derived from numerous tissue types including breast, prostate, renal, ovarian, colon, blood, lung, and central nervous system, revealed generally broad expression of YAP1 and WWTR1. Expression of LHX3 or ISL1 was low in each cell line, while expression of AJUBA was not available in these datasets. It should be noted that leukemia-derived cell lines had minimal expression of YAP1 and WWTR1, but this was matched by a lack of LHX3 or ISL1 expression. In line with our model, a subset of 6 NCI-60 cell lines able to poorly support entry of MCPyV pseudovirus were not permissive to “native” MCPyV infection (Schowalter *et al.*, 2012), indicating the presence of a post-entry restriction mechanism. It was previously

shown that KRT20-positive cells isolated from normal tissues could also not effectively support MCPyV pseudovirus entry or native MCPyV replication (Liu *et al.*, 2016). However only a total of 122 and 42 KRT20-positive cells, respectively, were assessed for each measurement due to the relative scarcity of Merkel cells in the skin. As a result, it is possible that a low sample size or inefficient entry prevented effective measurement of MCPyV infection kinetics in Merkel cells. Indeed, expression of ISL1 is exclusive to Merkel cells in the epidermis (Perdigoto *et al.*, 2014) and ISL1 and LHX3 expression is highly upregulated in Merkel cells relative to other epidermal cells (Haeberle *et al.*, 2004) concurrent with low expression of YAP1 (Rognoni and Walko, 2019). As such, normal Merkel cells represent a YAP1/WWTR1/AJUBA-low and LHX3/ISL1-high environment which our model predicts would support efficient MCPyV replication *in vivo*. In line with this idea, established MCCP cell lines which support efficient TAg expression, but not replication due to the omnipresent LT mutation, are LHX3/ISL1-high and YAP1/WWTR1/AJUBA-low (Figure 4.1). Indeed, although these established cell lines were unable to support robust entry of MCPyV pseudovirus, they were not assessed for MCPyV replication and virion production. Taken together, it is possible that transfection of MCPyV genomic DNA into Merkel cells or established MCCP cell lines is sufficient for efficient genome replication and virion production. Furthermore, it may be possible that co-transfection with LHX3 and ISL1 expression plasmids or knockdown or knockout of YAP1, WWTR1, or AJUBA could enhance MCPyV replication. Although these suggestions remain to be assessed, the potential for a viable and physiological MCPyV replication system through suppression of YAP1/WWTR1/AJUBA and upregulation of LHX3/ISL1 remains a tantalizing pathway for future investigations.

TP53- and NE-status as molecular targets for therapy in MCC

MCC is an aggressive, but poorly understood cancer with limited treatment options. Although localized disease can be managed through standardized surgery and adjuvant RT, metastatic MCC has traditionally been treated with an assortment of chemotherapeutic agents with minimal success (Becker *et al.*, 2017; Tanda *et al.*, 2021; Villani *et al.*, 2019). The recent advent of ICI immunotherapy has dramatically improved outcomes for patients with disseminated MCC (Kaufman *et al.*, 2016; Nghiem *et*

al., 2016), but ~50% of eligible patients do not respond to therapy (Tanda *et al.*, 2021). As such, improved therapeutics are needed to support this vulnerable patient population.

Current treatments for metastatic MCC are exclusively broadly acting and no established molecular markers which predict response to therapy or prognosis exist. Indeed, chemotherapy non-specifically induces DNA damage (Chabner and Roberts, 2005), while ICI immunotherapy broadly activates T-cell-dependent killing of tumor cells (Liu *et al.*, 2021). Furthermore, although MCCN and MCCP subtypes are genetically and etiologically distinct, responses to chemotherapy or ICI are generally considered synonymous (Handschei *et al.*, 2010; Kaufman *et al.*, 2016; Moshiri *et al.*, 2017; Nardi *et al.*, 2012; Nghiem *et al.*, 2016; Schrama *et al.*, 2011; Sihto *et al.*, 2009). However, it should be noted that the responses of MCCN or MCCP to specific chemotherapeutic agents was not assessed. In any case, clinical factors used to select treatment are non-molecular and depend on tumor location, patient health, and disease stage (Villani *et al.*, 2019). Stage IV MCC has an especially poor 5-year survival rate of 13.5%, as compared to 62.8%, 34.8-54.6%, or 26.8-40.3% for stages I, II, and III, respectively (Becker *et al.*, 2017), which is likely linked to the lack of MCC-targeted systemic therapy. Indeed, due to similarities in morphological presentation to other small-cell carcinomas, combined with the relative scarcity of MCC as compared to other skin cancers (0.6 and 25.3 cases per 100,000 per year for MCC and melanoma, respectively (Becker *et al.*, 2017; Saginala *et al.*, 2021)), metastatic MCC has traditionally been treated in an identical manner to other small-cell cancers, such as SCLC, with limited efficacy (Becker *et al.*, 2017). Thus, a critical step to enhance the treatment options available for disseminated MCC is the recognition and mechanistic validation of therapeutically-targetable biomarkers.

In this work, we identified *TP53*- and NE-status as potential molecular biomarkers to stratify MCC patients for targeted therapeutics. In Chapter 2, we demonstrated that *TP53*-wildtype MCC is preferentially sensitive to IMPDH inhibition. Mechanistically we found that IMPDH inhibition rapidly suppressed nascent DNA and not RNA synthesis to induce RS. The accumulation of RS subsequently triggered DDK-dependent activation of p53 and, ultimately, p53-dependent cellular cytotoxicity in MCC. Building on this mechanism, we utilized a novel combination of IMPDH and ATR inhibitors to induce cytotoxicity in *TP53*-mutant MCC through the induction of RC. With these key findings, we uncovered and validated the *in vitro* potential of targeted-therapeutic options for both *TP53*-wildtype and -mutant MCC. In

Chapter 3, we used bulk and single-cell transcriptomic and proteomic analyses to uncover previously unrecognized heterogeneity in NE gene expression within and between patient-derived tumor biopsies and cell lines. We observed a broad negative correlation of MCC-associated NE gene expression with that of Hippo pathway transcription factors YAP1 and WWTR1, similar to what is observed in SCLC. This reciprocal expression of a NE transcriptional program or YAP1 and WWTR1 allowed us to categorize MCC into novel NE-hi and NE-lo subtypes, respectively. Mechanistically, we observed expression of YAP1 or WWTR1 to suppress proliferation of NE-hi MCCP cells through the inhibition of MCPyV Tag expression and reinstatement of RB1-dependent cell-cycle dysregulation. In this work, we have identified NE-hi and -lo MCC subtypes which could be exploited for therapeutic benefit, as is the case for NE-based therapeutic strategies in SCLC. Taken together, these findings have several important implications as patient stratification markers for targeted therapy in MCC.

Our findings suggest that *TP53*-status is an important marker for identification of MCC patients sensitive to IMPDH inhibitors and, more broadly, chemotherapy. Indeed, it has already been speculated that wildtype *TP53* is essential for the anti-tumoral cytotoxicity of traditional chemotherapy through the activation of p53-dependent target genes which have growth-suppressive and pro-apoptotic properties (Ferreira *et al.*, 1999). In line with this idea, *TP53* is the most frequently mutated gene pan-cancer (Kandoth *et al.*, 2013). Indeed, we found that cellular death in response to IMPDH inhibition depended on functional p53 transcriptional activity (Figure 2.3). Beyond purely growth-suppressive and pro-apoptotic functions, p53 can also transactivate a plethora of other genes involved in diverse cellular processes including metabolism, inflammation, and autophagy to regulate cellular fate in response to stress (Kastenhuber and Lowe, 2017). For example, in response to DNA damage p53 can transactivate expression of ribonucleotide reductase subunit 2b (RRM2b) to provide additional dNTPs for repair of damaged DNA (Tanaka *et al.*, 2000). Indeed, *TP53* mutations are not purely inactivating and can often result in selection-of-function, loss-of-function, and gain-of-function alterations to normal p53 activity. These mutations often manifest as loss or selection away from p53-dependent growth suppression and apoptosis, while supporting more pro-oncogenic gene activation. In line with this idea, certain cancers can become dependent on mutant p53-mediated transcriptional activity to support survival and proliferation (Mantovani *et al.*, 2019). As a result, therapeutic activation of p53 through IMPDH inhibition or

chemotherapy may not induce cytotoxicity if *TP53* mutational status is not carefully determined and exploited. Indeed, *TP53* mutations were not evaluated in all prior retrospective analyses of chemotherapeutic responses in MCC (Iyer *et al.*, 2016; Tai *et al.*, 2000; Voog *et al.*, 1999), which may help to explain their suboptimal performance. Furthermore, selection of *TP53* mutant subpopulations upon treatment with chemotherapy has been demonstrated to rapidly promote resistance to therapy (Ferreira *et al.*, 1999; Lu and El-Deiry, 2009). In MCC, most *TP53* mutations are predicted to be inactivating (Knepper *et al.*, 2019; Starrett *et al.*, 2020), although the presence or importance of alternative *TP53* mutations has not been explored. As such, although our work has shown that *TP53* status is important for predicting sensitivity to IMPDH inhibition *in vitro* it is likely imperative to identify and monitor the functional characteristics of p53 *in vivo* throughout clinical course to ensure the effective exploitation of p53-dependent cytotoxicity.

Inhibition of IMPDH in the context of cancer therapy initially appears to have identical mechanistic consequences as traditional chemotherapy, which has noted toxicity in MCC patients. Our work has demonstrated that IMPDH inhibition activates p53-dependent cytotoxicity through the induction of RS and subsequent DNA damage due to the depletion of deoxyguanylate nucleotides (Figures 2.3, 2.5, 2.6, 2.7). Commonly used chemotherapeutics for the treatment of MCC, such as etoposide alone or in combination with carboplatin, are also known to induce p53 activation in conjunction with RS and DNA damage (Cheung-Ong *et al.*, 2013; Hientz *et al.*, 2017). However, the mechanism and order by which these events occur is distinct. Indeed, treatment with carboplatin or etoposide creates DNA adducts or entraps topoisomerase II onto DNA, respectively, to directly cause broad DNA damage which will subsequently induce RS and activate p53-dependent transcription (Cheung-Ong *et al.*, 2013). This initial and broad DNA damage often results in significant and dose-limiting toxicity through untargeted killing of normal cells (Schirrmacher, 2019). On the other hand, we have shown that IMPDH inhibition suppresses deoxyguanylate nucleotide levels to inhibit nascent DNA synthesis and sequentially induce RS and eventually DNA damage (Figures 2.5, 2.6, 2.7). As a result, IMPDH inhibition does not immediately incur severe DNA damage and this more indirect mechanism may result in a lower toxicity profile for IMPDH inhibitors. Indeed, the IMPDH inhibitor MMF (CellCept) is commonly used at high dosages (2-3g per day) in organ transplant patients with minimal side effects over several years of treatment (Mele and Halloran,

2000). MMF has been shown to prevent tissue rejection by inducing a cytostatic effect in inappropriately active and proliferating immune cells through the selective depletion of guanylate nucleotides (Allison and Eugui, 2000). Indeed, highly proliferative cells, including cancers, have an increased demand for nucleotides and must rely on the energy intensive *de novo* biosynthesis to support growth while nucleotide salvage pathways can usually provide sufficient nucleotide concentrations for normal, non-proliferating cells (Yin et al., 2018). Taken together, IMPDH inhibition has a distinct mechanism to induce p53-dependent cytotoxicity with a potential for lower dose-limiting toxicity as compared to traditional chemotherapeutics. As such, the utilization and development of more potent IMPDH inhibitors for use in *TP53* wildtype cancer should be further investigated.

Although our work has demonstrated that IMPDH inhibition induces p53-dependent cytotoxicity *in vitro*, the previous use of other chemotherapy for treatment of MCC has been thought to be minimally effective *in vivo*. Indeed, despite MCC being highly sensitive to chemotherapy (Becker *et al.*, 2017), the durability of responses are low (Iyer *et al.*, 2016) in conjunction with high toxicity-related deaths (Tai *et al.*, 2000; Voog *et al.*, 1999). As such, the utility of IMPDH as an additional chemotherapeutic agent initially appears minimal. However, the major retrospective studies which found chemotherapy poorly effective for the treatment of MCC did not determine *TP53* mutational status or the presence of MCPyV (Iyer *et al.*, 2016; Tai *et al.*, 2000; Voog *et al.*, 1999). Based upon the initial observation that 8 of 10 MCC tumors contained MCPyV DNA, it is broadly believed that up to 80% of MCC is MCCP (Feng *et al.*, 2008). Since *TP53*-mutational status and MCCP broadly overlap (Knepper *et al.*, 2019; Starrett *et al.*, 2020), most if not all MCCP are *TP53* wildtype. Critically, however, more recent estimates suggest percentages of MCCP closer to 50% (Knepper *et al.*, 2019; Starrett *et al.*, 2020). As a result, due to the lack of robust characterization of *TP53*-status in the retrospective analyses of MCC responses to chemotherapy it is uncertain whether *TP53* inactivation propends resistance to chemotherapeutic agents. As such, we suggest a more robust recognition and cataloging of *TP53*-status in patients to determine if chemotherapy or specific chemotherapeutic regimens propend survival benefits to *TP53*-wildtype MCC.

Although we have shown that the dominant effects of IMPDH inhibition are p53-dependent, the pronounced synergy in combination with ATR inhibition has clear clinical implications for MCC. Indeed, we have demonstrated that the cytotoxic effects of IMPDH inhibition could be enhanced upon

combination with ATR inhibition in cells without functional p53 (Figures 2.8, 2.9). Mechanistically, we demonstrated that this enhanced sensitivity depended on the “firing” of “dormant” origins via inhibition of ATR in the context of IMPDH inhibitor-induced RS which led to the exhaustion of cellular RPA levels and exposure of ssDNA to genome-wide DSBs which culminated in cell death independent of *TP53*-status (Figure 2.8, 2.9). Our findings are directly in line with other work utilizing DNA synthesis inhibitors in combination with ATR inhibition (Toledo *et al.*, 2013), albeit the use of ATR and IMPDH inhibitors in combination is novel. As such, although IMPDH inhibitor-based monotherapy would likely be ineffective in *TP53*-mutant MCC we have demonstrated the *in vitro* potential for combinatorial synergy with ATR inhibitors which could prove efficacious *in vivo*. Furthermore, although the relative potency of clinically available IMPDH inhibitors (CellCept) is relatively low (2-3g per day (Mele and Halloran, 2000)), combination with ATR inhibitors, which are under clinical assessment, could boost the anti-tumoral effectiveness for both *TP53*-based MCC subtypes. In addition, the combination of ATR inhibitors with traditional MCC chemotherapeutics could enhance their effectiveness, as is observed in numerous other cancers (Barnieh *et al.*, 2021). Currently, the use of ATR inhibitors in MCC have not been clinically assessed. Taken together, our work supports the translational development of synergistic IMPDH and ATR inhibitor treatments for broad use in MCC.

In addition to genetic *TP53*-based subtyping, our work has identified MCC subgroups based upon heterogenous expression of a NE transcriptional program (Chapter 3). Indeed, several therapeutic modalities currently under clinical assessment in SCLC are based upon the expression of phenotype-driving NE or non-NE transcription factors (Rudin *et al.*, 2019). In a strikingly similar manner, our work has demonstrated the broad separation of MCC into NE-hi and NE-lo subtypes based upon the reciprocal expression of a NE transcriptional program and Hippo pathway transcription factors YAP1 and WWTR1. As a result, the NE-based SCLC therapeutics could be rapidly leveraged for use in MCC based upon our novel NE-defined subtypes.

The NE-hi/ASCL1-positive SCLC subtype overexpresses delta-like ligand 3 (DLL3), an inhibitory Notch-pathway ligand that is similarly highly expressed in other NE cancers and minimally in normal tissues or the other non-NE SCLC subtypes (Owen *et al.*, 2019). DLL3 was also found to be overexpressed in a subset of MCC tumor samples (Xie *et al.*, 2020), an effect mirroring our NE-hi and

NE-lo MCC subclassifications. Early efforts to target DLL3 in SCLC have focused on rovalpituzumab tesirine (Rova-T), an anti-DLL3 antibody conjugated to pyrrolobenzodiazepine (PBD), a DNA-binding and crosslinking agent, along with a protease-cleavable linker (Owen *et al.*, 2019). It is thought that upon binding to DLL3 the Rova-T:DLL3 complex is endocytosed, allowing for subsequent lysosome-dependent linker cleavage and release of PBD. The freed PBD then transits to the nucleus and incurs DNA damage specifically in DLL3-expressing cells. Despite effective control of DLL3-expressing SCLC *in vitro* and *in vivo* (Saunders *et al.*, 2015), several large scale clinical trials showed low ORR (9-14.3%) and PFS (3-4.1 months) after treatment (Blackhall *et al.*, 2021; Johnson *et al.*, 2021; Morgensztern *et al.*, 2019). As a result, development of Rova-T was discontinued by its respective company (AbbVie). It should be noted that several of these studies included unselected patients with DLL3-low (>50%) or non-expressing tumors as measured by IHC which composed 42.0-58.8% of the total test population (Johnson *et al.*, 2021; Morgensztern *et al.*, 2019). In addition, none of these studies performed longitudinal tumor biopsies to determine if DLL3 expression was sustained over the course of treatment, or to assess the potential for escape mutants. These results further emphasize the importance of identifying proper patient populations and following these patients throughout treatment to ensure effective use of targeted treatments.

A single MCC patient has been treated with Rova-T after becoming refractive to both chemotherapy and ICI (Xie *et al.*, 2020). In this patient, it was reported that 30% of the tumor biopsy contained DLL3-expressing cells. Strikingly, significant decreases in the sizes of the patient's tumor lesions (57%) indicated the potential for efficacious treatment of MCC. Indeed, perhaps modification of the cytotoxic entity, dosages, and treatment schedule could improve efficacy of Rova-T in SCLC as well as MCC.

Other emerging modalities targeting DLL3 are also being assessed for clinical relevance in SCLC. These include anti-DLL3 chimeric antigen receptor T-cells (AMG119) and bispecific DLL3-cluster of differentiation 3 (CD3) antibodies (AMG757) (Owen *et al.*, 2019). Instead of inducing cytotoxicity through payload delivery, these modalities redirect cytotoxic T-cell activity to DLL3-expressing cells to control tumor growth. Both AMG119 and AMG757 are currently being clinically assessed (NCT03392064 and NCT05060016, respectively), though no results have been published. With careful consideration and

monitoring of NE-status, the application of these DLL3-specific modalities could potentially be bridged for therapeutic effect in NE-hi MCC.

In addition to NE-hi/DLL3-based treatments, therapeutic targeting of NE-lo/YAP1/WWTR1-based SCLC has emerged. It has recently been demonstrated that the YAP1 SCLC subtype is sensitive to cellular death mediated via ferroptosis while the ASCL1 subtype is insensitive (Bebber et al., 2021). Ferroptosis is an emerging mechanism of program cell death which relies on iron-dependent accumulation of lipid reactive oxygen species (ROS) (Li et al., 2020). Chemical inhibition of glutathione peroxidase 4 (GPX4), responsible for converting cytotoxic lipid peroxidases to corresponding alcohols, resulted in the accumulation of ROS and subsequent induction of ferroptosis. Indeed, treatment with a GPX4 inhibitor preferentially sensitized YAP1 SCLC to ferroptosis and cellular death while not significantly effecting the ASCL1 subtype (Bebber *et al.*, 2021). Although clinical efficacy of ferroptosis-activators remains to be shown, the potential usage in our NE-lo/YAP1/WWTR1-hi MCC subtype represents an attractive target for cross-application in future studies.

In addition to stratification for targeted therapy, it has been suggested that NE and non-NE SCLC subtypes have distinct tumor microenvironments with prognostic implications for currently used therapeutics. Indeed, examination of SCLC patient tumor biopsies (n=32) revealed increased CD3⁺ and CD8⁺ T-cell tumor infiltration of primary tumors and lymph node metastases in the non-NE subtype as compared to NE (Dora et al., 2020). It is possible that this increased level of immune cell infiltration can explain the beforementioned enhanced survival of patients with the non-NE verses NE SCLC subtype (Qi *et al.*, 2022), since increased immune cell infiltration is broadly associated with better outcomes pan-cancer (Barnes and Amir, 2017). Furthermore, the YAP1 SCLC subtype has also been characterized as an “inflamed” subtype due to higher expression of immune-associated genes including cytotoxic T-cell markers such as CD8A/B, various human leukocyte antigen (HLA) and processing machinery, interferon- γ -associated genes, and PD-L1 and PD-1 (Gay et al., 2021). In the same report, this inflamed phenotype had increased sensitivity to ICI relative to the other SCLC subtypes although it should be noted that these results were simple trends since the study was not sufficiently powered for prediction of significance. Interestingly, assessment of our patient-derived tumor biopsy RNA-seq revealed a predisposition in the NE-lo/YAP1/WWTR1 subtype for expression of immune-infiltrating markers with

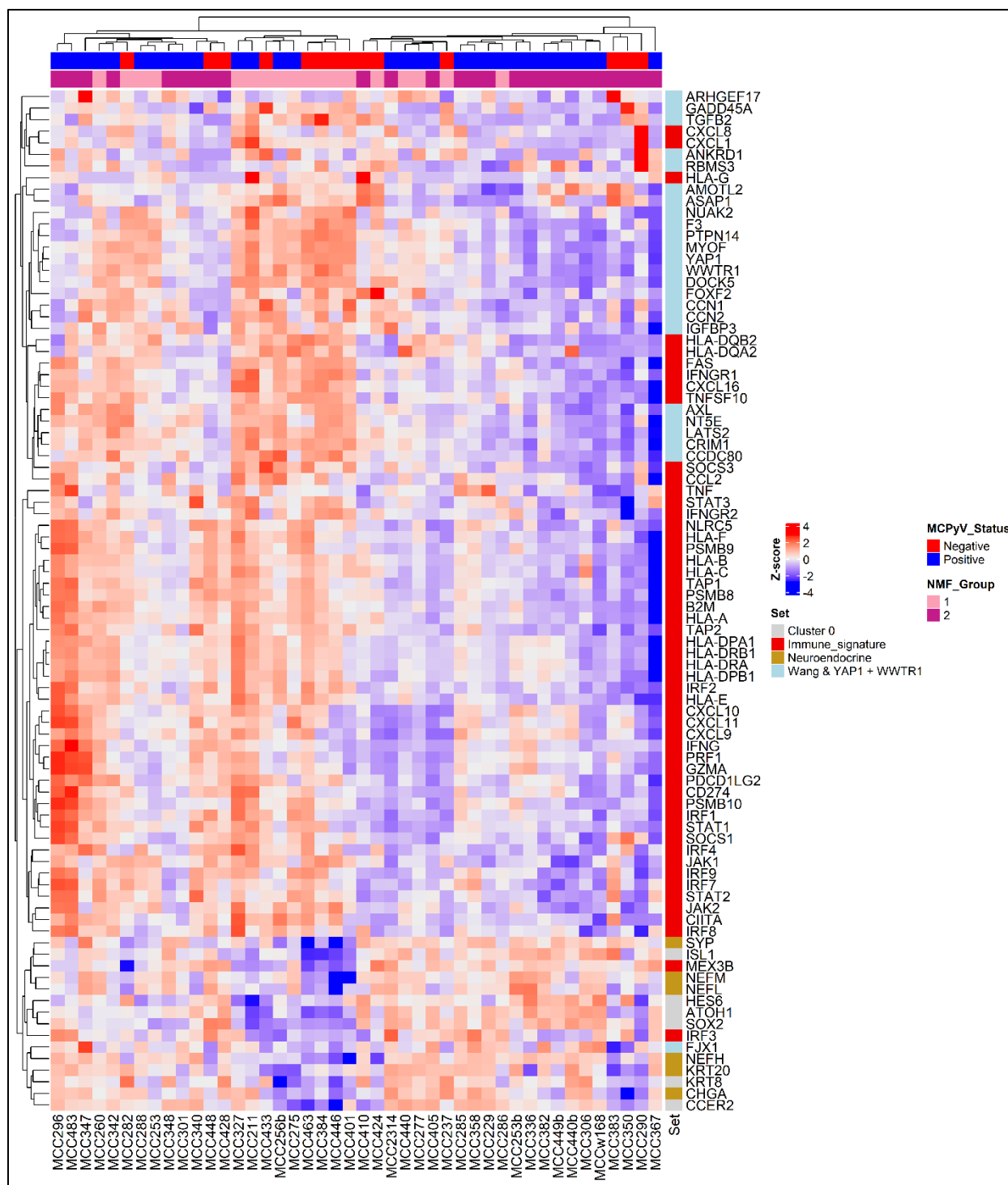


Figure 4.6 NE-status in MCC is inversely correlated with expression of immune-associated genes. RNAseq analysis of patient-derived tumor biopsies as in Figure 3.3 but with inclusion of immune-associated genes from Gay et al. Row colors denote gene set affiliation. Column colors represent NE status (NMF_1 is NE-lo and NMF_2 is NE-hi) and MCPyV status (red = negative, blue = positive). Analysis was performed as in Figure 2.3 but with additional genes added from Gay et al.

some intermingling with a subset of the NE-hi subtype (Figure 4.6). As a result, it is possible that ICI-non-responders in MCC are enriched for the NE-hi subtype, through this remains to be confirmed in clinical datasets. Interestingly, scRNA-seq analysis revealed the emergence of an “inflamed”/YAP1 SCLC cell population that correlated with cisplatin resistance in an ASCL1-dominant *in vivo* model (Gay *et al.*, 2021). As such, it is possible that prior chemotherapeutic treatment may drive SCLC, and possibly MCC, into a YAP1- and WWTR1-driven transcriptional state which is more responsive to ICI. Taken together, our identification of NE subtypes in MCC remains an emerging but clinically relevant marker for patient stratification.

Conclusions

Our work has expanded the molecular characterization of MCC to encompass several novel and therapeutically-relevant subtypes and unlocked the potential cell-of-origin for MCC. Based upon genetic inactivation of *TP53* or the relative expression of NE genes in MCC, our work supports the use of clinically available therapeutic modalities for targeted treatment of these subtypes. While admittedly our work remained mostly *in vitro*, we believed that it has served as a mechanistic foundation from which the *in vivo* potential of IMPDH or ATR inhibitors and NE-targeting biologics can be explored. Through the lens of our stratification scheme, it has become abundantly clear that monitoring the genetic and expressional landscape of MCC tumors throughout clinical course is critical for effective treatment. Indeed, much of the effectiveness of our proposed stratifications and cognate treatments depend on the accurate identification and continued expression of these markers. Although not all our suggested markers are commonly assessed in the clinic, they are readily observable with commonly used techniques including IHC and targeted sequencing. A major obstacle to be overcome is the definition of NE-hi and NE-lo tumor subtypes within a clinical setting. As such, we recommend medical providers to inform and collect patient tumor samples and metadata to support the relative scarce and incomplete treatment, genetic, and expressional datasets currently present within the MCC field. In addition to assisting our work, the better collection of these data will surely unlock other targeted therapeutic vulnerabilities which are otherwise hidden within current analyses. Our work has also uncovered a model for the cell-of-origin for MCCP and suggested a transcriptional environment permissive for production of MCPyV virions. Indeed, similar to

HPV, we suggest that MCPyV initially infects proliferative basal epidermal cells and lies dormant until differentiation into Merkel cells allows a transcriptional environment conducive for replication. Thus, integration of MCPyV into normal Merkel cells is the likely oncogenic mechanism for MCCP. In terms of MCCN cell-of-origin, it is possible that proliferative basal epidermal cells can similarly support their transformation. However, due to the high mutational burden present in most MCCN, the potential for a mutation-induced NE phenotype is possible from a broad pool of cells. In any case, the work we have described here has made significant contributions for the field of MCC and other NE cancers. We hope these findings can be used translationally to enhance patient outcomes and progress towards a world with readily treatable cancer.

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