SUPPLEMENTARY MATERIALS AND METHODS

**Antibodies**

Antibodies used for immunoblot and immunohistochemical (IHC) analysis include the following: p53 (NCL-p53-CM1 from Novocastra, UK); cleaved Caspase-3 (cCasp3; No. 9662 for immunoblotting; No. 9661 for immunohistochemistry), cleaved PARP (cPARP; No. 5625), Bad (No. 9292), Bmf (No. 4692), Bik (No. 4592) and Bcl-w (31H4, No. 2724 from Cell Signaling Technology, MA); Bcl-2 (clone 124, No. M0887 from Dako, Denmark); Bim (IMG-171 from Imgenex, CA); Bcl-xL (No. 610212 from BD Biosciences, CA); Bax (No. 06-499), Bak (No. 06-536), Noxa (OP-180, No. 114C307), Ki67 (No. MAB4190), and phospho-HistoneH3 (pHisH3; Clone RR002, No. 05-598 from EMD Millipore Corp, MA); MCPyV LTAg (CM2B4, sc-136172), PUMAα/β (G-3, sc-374223), and Mcl-1 (S-19, sc-819 from Santa Cruz Biotechnology, CA); survivin (No. NB500-201 from Novus Biologicals, CO); Keratin 20 (K20; EPR1622Y, ab76126), synatophysin (Syn; YE269, ab32127), neuron specific enolase (NSE; ab39369), and thyroid transcription factor 1 (TTF1; EP1584Y, ab76013 from Abcam, MA); Keratin 8 (K8; Troma I from Developmental Studies Hybridoma Bank); and Chromogranin A (CgA; SP-1, No. 20086 from Immunostar, WI). Anti-α-tubulin (No. T5168) or anti-β-actin (No. A5316 from Sigma-Aldrich, MO) was routinely included as a loading control.

**Establishment and culture of UM-MCC cell lines from human tumors**

Although it is now clear that Merkel cells are derived from epidermal progenitors (Morrison et al., 2009; Van Keymeulen et al., 2009; Woo et al., 2010) at the time this project was initiated Merkel cells were believed to be derived from the neural crest (Szeder et al., 2003). As a result, we fortuitously initiated and maintained MCC cultures in a modified neural crest stem cell self renewal medium (Molofsky et al., 2005) consisting of 50% low glucose DMEM and 30% neurobasal medium...
(Gibco) supplemented with 15% chick embryo extract (Stemple and Anderson, 1992), 50µM 2-mercaptoethanol, 35ng/mL retinoic acid (Sigma), 1% N-2 and 2% B-27 Supplements (Invitrogen), 20ng/mL basic fibroblast growth factor and 20ng/mL insulin growth factor (R&D Systems, MN), 1% non-essential amino acids (Gibco), 100U/mL penicillin/streptomycin and 0.50µg/mL fungizone (Invitrogen). All UM-MCC cell lines were established in culture either directly from patient MCC tumors or from tumors arising in NOD/SCID/IL2Rγnull (NSG) mice by finely mincing solid tumors or dissociation into single cells followed by seeding in 12-well tissue culture plates as specified in Table 1.

During establishment, cultures were monitored by optical microscopy and initially supplemented with fresh media as cell aggregates expanded. After 1-2 weeks, large spheres and clusters were vigorously triturated to physically separate them into smaller aggregates. MCC cell lines were initially passaged 1:2 to maintain a high cell density, or half the medium was removed and replaced with fresh medium every 3-5 days as needed. Establishment of cell lines was quite variable with some lines such as UM-MCC29 highly proliferative at the outset while others such as UM-MCC13 were slower growing. Proliferation rates were noted to increase in the majority of cell lines once higher passages were reached with doubling times of typically 1-2 days (Suppl. Table S1). Once established, the majority of cell lines could be routinely passaged 1:5 or higher. Single cell counts were performed using a hemocytometer following trypsinization with 0.05% trypsin-EDTA (Gibco). Trypsinization of cell lines growing as tight spheres often yielded extracellular debris that was removed by filtration through a 70µM filter.

Initial establishment of cell lines directly from human MCC tumors yielded a 39% success rate, while 3 of 4 MCC tumors grown in NSG mice yielded genuine MCC lines. All UM-MCC lines have been passaged over 60 times and maintained in culture for at least 18 months at the time this
manuscript was submitted. Initially 13 cell lines were established from primary or metastatic human MCC tumors collected from 11 patients, however, 2 lines were lost to contamination after months in culture, dropping the final count to 11 UM-MCC cell lines from 9 patients (Table 1, Suppl. Table S1). Similar to most previously characterized bona fide MCC cell lines, all UM-MCC lines grow in suspension as tight spheres or loose clusters (Suppl. Fig. S1a). Although several adherent cell lines established from MCC tumors have been described (Leonard et al., 1995), none of the UM-MCC lines demonstrated adherence and the few MCC tumors that grew as monolayers were discarded since they did not express typical MCC markers. All cell lines were screened for mycoplasma contamination using the Mycoprobe Kit (R&D Systems).

**RNA and Protein Analysis**

Expression of human *atonal homolog 1 (ATOH1)* (GenBank NP_005163) to validate cells of Merkel cell lineage was assessed using primers previously reported (Harms et al., 2013) and expression of *Keratin 20 (K20)* (GenBank NM_019010) was performed using: forward primer targeting exon 6 nts: 1083-1102, reverse primer targeting exon 7 nts: 1261-1280 (shown in Suppl. Fig. S1b). Briefly, RNA isolation was carried out with UM-MCC cell lines using the Qiagen RNeasy mini Kit as per manufacturer’s instructions, and cDNA synthesis prepared with SuperScript™ II Reverse Transcriptase (Invitrogen) using 0.25μg RNA. Cell line characterization included assessment of multiple MCC markers by immunoblotting for simple epithelial K20 and K8 as shown in Suppl. Fig. S1c. K20 staining in the typical paranuclear dot-like pattern was further assessed by embedding cell line aggregates in HistoGel specimen processing gel (Richard-Allan Scientific). Briefly, non-trypsinized cell clusters were fixed in cold methanol, washed in PBS and resuspended in warmed HistoGel which was then cooled to allow solidification of the gel pellet for routine paraffin embedding,
processing and IHC staining (shown for UM-MCC31; -34, and -565 in Suppl. Fig. S1d). Matched human MCC tumors from which cell lines were derived also stained with K20 to reveal a typical staining pattern. UM-MCC cell lines negative for K20 protein displayed similar negativity in initial MCC tumors from which they were derived. Antibodies directed against CgA, Syn and NSE were used as neuroendocrine markers (shown in Suppl. Fig. S1c). Melanoma cell lines SK-Mel-19 and -103 are shown as controls for transcript and protein expression analysis. TTF1, a marker of small cell lung cancer cells (Cheuk et al., 2001), was not detected in UM-MCC cell lines (data not shown). A summary of the UM-MCC cell line panel characterization, based on gene and/or protein expression of simple epithelial keratins and neuroendocrine markers, is shown in Suppl. Table S1.

**MCPyV status in UM-MCC cell lines**

_Semi-quantitative PCR:_ RNA and DNA were isolated from UM-MCC cell lines using the Qiagen RNeasy mini Kit or the DNeasy Blood and tissue Kit, respectively, as per manufacturer’s instructions. cDNA synthesis was prepared with SuperScript™ II Reverse Transcriptase (Invitrogen) as described above. Detection of TAg sequences (GenBank NC_010277) was assessed by semi-quantitative PCR on cell line cDNA and/or genomic DNA. Primers used include TA2 (Harms et al., 2013) targeting exon 1 forward (F)-primer nts: 354-373 and reverse (R)-primer targeting coding region specific to sTAg only nts: 571-590; TA3 targeting exon 2 F-primer nts: 1054-1073 and R-primer nts: 1198-1208 resulting in a 155bp TAg transcript or PCR product. Combining TA2 F-primer targeting exon 1 and TA3 R-primer targeting exon 2 allowed detection of a specific LTAg transcript (424bp) with intronic sequence spliced out and a TAg PCR product of 855bp. Specific human β-actin primers were used as a control. PCR products were separated by agarose gel electrophoresis and visualized by Midori Green DNA stain (Nippon Genetics) (Suppl. Fig. S2a, c). Melanoma lines SK-Mel-19 and SK-
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Mel-147 as well as foreskin isolated human melanocytes and fibroblasts were used as controls (data not shown).

**Quantitative real-time PCR:** Isolated RNA (0.5 µg) was utilized for first strand cDNA synthesis with SuperScript™ III Reverse Transcriptase (Invitrogen) as described above. Quantitative PCR reactions were performed on the StepOne real-time PCR system (Applied Biosystems) using Taqman detection. For detection of LTAg, the following previously reported primer/probe sets were used: SET6, SET7, LT2 (Rodig et al., 2012) and TAg (Shuda et al., 2009). For detection of sTAg, the following previously reported primer/probe sets were used: LT3, SET9 (Rodig et al., 2012) and sTAg (Bhatia et al., 2010). Input genomic DNA was 15 ng (SET6, SET7, SET9, LT2, LT3) or 50 ng (TAg, sTAg) according to the input amount specified in the report for that primer/probe set. GAPDH (Assay ID Hs99999905_m1, Life Technologies) or RNaseP TaqMan® Copy Number Reference Assay (catalog #4403326, Invitrogen) were used to normalize results for cDNA and genomic DNA, respectively. All samples were run in duplicate. Results were analyzed using the $2^{-\Delta\Delta CT}$ method. For copy number estimation, qPCR results from genomic DNA were compared to a standard curve established from serial dilutions of MKL-2 genomic DNA (previously reported to have 1 MCPyV copy/genome) (Rodig et al., 2012). Fold increase in TAg transcript expression was also normalized to MKL-2 cells (Suppl. Fig. S2d).

**Immunoblotting and Immunohistochemistry:** Further evaluation of MCPyV LTAg protein levels in the UM-MCC cell lines as assessed by immunoblotting are shown in Fig. 4b. Additionally, we evaluated the status of MCPyV LTAg expression in the human MCC patient tumors from which the UM-MCC cell line panel was established by both IHC staining and immunoblotting using tumor lysates with the CM2B4 LTAg antibody as previously described (Rodig et al., 2012), revealing patient
tumor numbers 2, 4, and 9, corresponding to UM-MCC lines 13, 29, and 565, were positive for expression of LTAg (not shown).

MCPyV status for the current UM-MCC cell line panel based on semi-quantitative PCR, RT-PCR and immunoblotting is summarized in Suppl. Table S1. Results from these experiments confirm that UM-MCC13, -MCC29, and -MCC565 are MCPyV-positive. Additionally, the 2 lost lines UM-MCC19 and -MCC49 were confirmed MCPyV-positive based on the expression of LTAg transcripts and protein (data not shown) and are included in Suppl. Table S1. The remaining MCC cell lines designated as MCPyV-negative express TAg RNA and DNA at levels that are typically several orders of magnitude lower, in the same range as negative control cell lines, and are therefore not scored as true positives. Taken together, our data indicates 5 MCPyV-positive MCC cell lines were initially established from 11 patient tumors (45% MCPyV-positivity). While we do not have an explanation for the relatively low prevalence of MCPyV in the University of Michigan MCC tumor cohort, several other studies have also reported lower incidence ranging from 24-100% ((Garneski et al., 2009; Leitz et al., 2013) and citations within).

**Analysis of cell proliferation**

Doubling times (T_d) for UM-MCC cell lines were estimated at the passage indicated (shown in Suppl. Table S1). Following trypsinization into single cells, and a 48h incubation period to allow cells to reform distinctive spheres or clusters and ensure exit from lag growth phase, doubling times were calculated based on proliferation during log growth phase by the XTT assay. A cell number titration curve at 3 consecutive 24h intervals was performed to ensure linearity of growth phase and assay measurements. All cells were seeded in triplicate in 96-well plates.
**Tumorigenicity of UM-MCC cells**

To assess tumorigenic potential, trypsinized cell lines were counted and $1.0 \times 10^6$ UM-MCC cells were resuspended in 50% Matrigel and injected subcutaneously in rear flanks of athymic mice. The majority of xenograft tumors were palpable at approximately 3-4 wks. Harvested mouse xenograft tumors were analyzed by hematoxylin and eosin to assess basic morphological features characteristic of MCC tumors. Expression patterns of keratins, neuronal markers, and MCPyV LTAg were analyzed by immunoblotting or IHC staining as described for the UM-MCC cell lines and human MCC tumors. All of the UM-MCC cell lines tested (9 out of 11) are tumorigenic in nude mice (Table 1), with data for the representative UM-MCC29 cell line shown in Suppl. Figure S3.
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**SUPPLEMENTARY FIGURE S1. Establishment and characterization of UM-MCC cell lines: lineage markers analysis.**

a) Microphotographs of newly established UM-MCC cell line panel. All lines grow in suspension as tight spheres or loose clusters. Scale bars = 50 μm.

b) Semi-quantitative RT-PCR for expression of *atonal homolog 1* (*ATOH1*), and Keratin 20 (*K20*) mRNA in the UM-MCC cell line panel compared to control melanoma cell lines SK-Mel-19 and -103. Actin is used as a control.

c) Protein expression levels of synatophysin (Syn), K20, Keratin 8 (K8), chromogranin A (CgA), and neuron specific enolase (NSE). Tubulin is a loading control.

d) Staining pattern in representative UM-MCC cell lines following suspension in Histogel, paraffin embedding and processing followed by immunohistochemistry for K20. Hematoxylin is used as a counterstain. Scale bars = 25 μm.
**Supplementary Table S1. Summary of UM-MCC cell line characteristics.** Summary of data used to characterize the newly established UM-MCC cell line panel as shown in Suppl. Fig. S1-S2. All other acquired data is explained in Supplementary Materials and Methods. “+” denotes positive; “−” denotes negative; “±” denotes weak expression; K20 = Keratin 20; ATOH1 = atonal homolog 1; sTAg = MCPyV small T antigen; LTAg = MCPyV large T antigen; K8 = Keratin 8; CgA = chromogranin A; NSE = neuron specific enolase; Syn = synaptophysin; Td(h) = doubling time in hours; ND = not determined. * indicates MCC lines lost to contamination (data not shown for these lines).

| UM-MCC cell line | Est. Passage | Est. Td (h) | K20 | ATOH1 | sTAg | LTAg | K8 | CgA | NSE | Syn |
|------------------|--------------|-------------|-----|-------|------|------|----|-----|-----|-----|
| 9                | ND ND        | ND          | +/- | +/ND  | -/ND | +/- | ND | +   | ND  | +   |
| 13               | ND ND        | ND          | +/- | +/ND  | +/ND | +/- | ND | +   | ND  | +   |
| 19*              | ND ND        | ND          | +/- | +/ND  | +/ND | +/- | ND | +   | ND  | +   |
| 29               | 150 27       | -/-         | +/ND | +/ND  | +/- | +/- | ND | +   | ND  | +   |
| 31               | 115 32       | +/-         | ND  | ND    | +/- | +/ND | +/- | +   | ND  | +   |
| 32               | 75 35        | +/-         | +/ND | +/- | +/- | +/ND | +/- | +   | ND  | +   |
| 34               | 45 28        | +/-         | +/ND | +/- | +/- | +/ND | +/- | +   | ND  | +   |
| 35               | 100 29       | +/-         | +/ND | +/- | +/- | +/ND | +/- | +   | ND  | +   |
| 40               | 140 33       | +/-         | +/ND | +/- | +/- | +/ND | +/- | +   | ND  | +   |
| 49*              | ND ND        | +/-         | ND/ND | ND/ND | +/- | ND/ND | +/- | ND  | ND  | +   |
| 565              | 60 36        | +/-         | +/ND | +/- | +/- | +/ND | +/- | +   | ND  | +   |
| 623              | 100 34       | +/-         | +/ND | +/- | +/- | +/ND | +/- | +   | ND  | +   |
| 624              | 125 32       | +/-         | +/ND | +/- | +/- | +/ND | +/- | +   | ND  | +   |
SUPPLEMENTARY FIGURE S2. MCPyV DNA and RNA status in UM-MCC cell lines. a) Semi-quantitative PCR or b) quantitative real time (QRT)-PCR analysis for the presence of TAg viral DNA sequences in the UM-MCC cell line panel. K20 is shown as a control in a). Control cell lines include MKL-1, MKL-2, HEK293, melanoma SK-Mel-147, and colon adenocarcinoma HT29. MCPyV genome copy number in b) was calculated assuming that MKL-2 cells contained 1 copy of viral DNA per cell. c) Semi-quantitative PCR and d) QRT-PCR for RNA expression of MCPyV TAg transcripts. Control cell lines include MKL-1, MLK-2, and HEK293. QRT-PCR was analyzed using the $2^{-\Delta\Delta CT}$ method and results are shown normalized to MKL-2. All primers are described in Supplementary Materials and Methods.
**SUPPLEMENTARY FIGURE S3.** Tumorigenicity of representative UM-MCC29 cell line in vivo.

Top: H&E, synaptophysin and MCPyV LTag IHC staining for patient tumor No. 4 which gave rise to cell line UM-MCC29; Middle: gross photos (d36) and growth kinetics following subcutaneous injection of 1.0x10⁶ cells; Bottom: matched IHC staining from xenograft tumor. All scale bars = 25 µm.
SUPPLEMENTARY FIGURE S4. Marginal effects of genetic inactivation of Bcl-2 in UM-MCC29. a) Fluorescent microphotographs are shown at times indicated following infection of UM-MCC29 with lenti-viral driven control (shCon) and Bcl-2 (shBcl-2) shRNA constructs. GFP fluorescence confirms shRNA expression. Scale bars = 50 µm. b) Downregulation of Bcl-2 is validated by immunoblotting. c) Quantitative analysis is determined as the reduction in number of live shBcl-2 expressing cells compared to shCon cells determined by Trypan blue exclusion assays. Error bars indicate SEM.