Lee et al Supplementary Material

SUPPLEMENTARY METHODS

Immunoblotting and immunoprecipitation.

Cells were lysed in NETN buffer [250 mM NaCl, 0.5 % NP-40, 20 mM Tris·HCl (pH 7.4), 1 mM EDTA, 25 mM NaF, 0.1 mM Na_3VO_4, 25 mM □-glycerophosphate, 1 mM DTT and a 1:100 dilution of Protease Inhibitor Cocktail (P8340, Sigma, Sydney, Australia)]. For p16 immunoprecipitates, cell lysates were precleared, immunprepcipitated with p16 antibody the immunoblotted as described previously [1]. Protein bands were visualised by enhanced chemiluminescence.

Immunohistochemisty.

Tissue microarray (ME1004a; Biomax, Rockville MD) from formalin fixed and paraffin embedded; benign naevi (24) and Stage II cutaneous melanomas (56), lymphatic and distant metastasis (20) each lesion is represented by a 2 mm core. TMA sections were prepared and immunostained as described previously [2]. Staining intensities and proportions were scored by three assessors, one of whom is a pathologist, to produce consensus scores. Staining intensities of sections were scored using a discrete scale of 0-4 for intensity, and percentage of cells stained on a scale of 0-3 (0, <33%, <66%, >66%). The product of both scores served as the final histochemistry score (H-score).

siRNA transfections

Melanoma lines D22, D28 and MM383 were seeded in T25 flasks, and transduced 2 h later with either empty vector or $CDK4^{R24C}$ virus. Two days later, they were harvested by trypsinisation and used for reverse transfection in 384 well plates, using ON-TARGETplus Smart Pool siRNA reagents (20 nM) and Dharmafect2 (Thermo Scientific Dharmacon)

transfection reagent (0.2% in OPTIMEM) as described previously[3]. The plates were fixed 3 days after transfection and stained with DAPI and anti-Ki67 antibody as above. EdU incorporation measurement was as reported previously [4].

Senescence bypass in MDA-MB-231 and SKMel 13.

SK-Mel-13 cells [5] were screened using the same conditions as the primary screen. MDA-MB-231-TetR-CMV/TO-p16 cells with tetracycline-inducible p16 WT were previously established as described [6]. MDA-MB-231-TetR-CMV/TO-p16 clone 1 cells used for tertiary screening were cultured in DMEM supplemented with 10 % Tet System Approved foetal bovine serum (Clontech, Mountain View, CA, USA), 4 mM L-glutamine, 1 mM sodium pyruvate and 10 mM HEPES. Assay was similar to the primary screen except that 1000 ng/ml doxycycline hyclate; DOX (Sigma, Sydney, Australia) was used for p16 induction instead of IPTG.

Senescence-associated -galactosidase Assay and Colony Formation Assay

Senescence associated- -galactosidase (SA- -gal) assays were performed essentially as previously described by Dimri et al {Dimri, 1995 #27}. Cells were washed fixed for five minutes at room temperature in 2 % paraformaldehyde and 0.2 % glutaraldehyde in PBS. Cells were then incubated overnight for 12-16 hours in fresh SA-β-gal staining solution [17.9 ml 0.1 M citric acid/32.1 ml 0.2 M Na_2HPO_4 buffer (pH 6.0), 150 mM NaCl, 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ and 20 mg/ml 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (Sigma, Sydney, Australia)]. Images of SA- -gal stained cells were taken under phase-contrast microscopy. For colony formation assays, cells were plated at 1000 cells/10 cm dish. Colonies were visualised by fixing cells for five minutes in ice-cold methanol, staining with Crystal Violet staining solution (0.25 % Crystal Violet and 25 %

ethanol) for 5 min and then de-stained by washing twice with distilled water. Dishes were imaged using a flatbed scanner and colonies were counted.

References:

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4 Ranall, M. V., Gabrielli, B. G. & Gonda, T. J. Adaptation and validation of DNA synthesis detection by fluorescent dye derivatization for high-throughput screening. *Biotechniques* **48**, 379-386 (2010).

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Table S1. Primary screen analysis of well data

Table S2. Secondary screen Ki67 individual well data.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1.

Functional assessment of a senescent phenotype in WMM1175 p16 WT clone B17 cells.

a) Immunoblots for p16 and a-tubulin in cells cultured under the following conditions: absence of IPTG, presence of IPTG for 5 days, continual presence of IPTG for 19 days or the presence of IPTG for 5 days followed by its absence for 14 days. The MDA-MB-468 sample served as a control for p16 expression under an endogenous promoter. **b)** Crystal-violet-stained plates from the colony formation assay after p16 induction by IPTG. Cells were treated with IPTG for five days and then cultured for 14 days with or without IPTG and compared to untreated cells. **c)** The cytoplasmic area of WMM1175 cells at 5 days with or without after p16 induction at different plating densities. **d)** The Z' scores calculated for Ki67 and cytoplasmic area.

Figure S2 Image analysis data-processing steps.

1. Adjustment of Ki67 fluorescence-intensity values (log-transformed) for each scored nucleus. Values were normalisaed so that density plot modes were aligned to compensate for variability between screening batches of plates (similar adjustment was performed for DNA and GFP channels), **2.** Thresholds were set using frequency distribution for untransduced (mock) wells labelled only with secondary antibodies to assign GFP status, Ki67 status (shown), and cell cycle-phase. **3**. Proportion of Ki67 positive of GFP positive cells was calculated as illustrated in the whole-screen well-data scatterplot showing percent Ki67 positive cells (y-axis) with plates (x-axis) grouped by screening batch . **4.** Plate median-based Z-scores (y-axis) were used to normalise data, and hit-picking cut off selected using positive (red-CDK4^{R24C}) and negative (yellow-vector) controls as a guide.

Figure S3.

Detection of p16 by immunocytochemistry during secondary screen performed in two series of plates. Bar graphs correspond to data in Table S2. All wells have IPTG added to induce

p16 expression. Mouse anti-p16 on the triplicate set of plates (Bar = mean, error bar =SD, n=3).

Figure S4.

Immunoblots of lysates from a panel of melanoma cell lines immunoblotted for CDK6, using -tubulin as loading controls.

Figure S5.

Effect of CDK6 or HMGB2 knockdown in melanoma cell lines differing in p16 genotype and endogenous CDK6 levels. **a)** Melanoma cell lines D22, D28 and MM383 were transfected with, with non-targeting (NT), CDK6 or HMGB2 siRNA. At 3 days after transfection, cells were pulsed with EdU for 2 h, then fixed and stained for EdU incorporation. The data are the mean and SEM from 4 wells. **b)** Melanoma cell lines D22, D28 and MM383 were transduced with empty vector or $CDK4^{R24C}$ expressing lentivirus, then transfected with lipofection reagent alone (untransfected), with non-targeting, CDK6 or RNA polymerase 2 siRNA. The latter gene depletion is toxic and was used as an indicator of transfection efficiency. At 3 days after transfection, cells were fixed and stained with DAPI, and total cell number assessed. These data are the mean and SEM from 4 wells. **c)** Melanoma cell lines transfected as in **a**) were immunoblotted and quantified for CDK6 depletion.