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Loss of *CDKN2A* Expression is a Frequent Event in Primary Invasive Melanoma and Correlates with Sensitivity to the CDK4/6 Inhibitor PD0332991 in Melanoma Cell Lines

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Summary

We have investigated the potential for the p16-cyclin D-CDK4/6-retinoblastoma protein pathway to be exploited as a therapeutic target in melanoma. In a cohort of 143 patients with primary invasive melanoma we used fluorescence in situ hybridisation to detect gene copy number variations (CNVs) in *CDK4, CCND1* and *CDKN2A* and immunohistochemistry to determine protein expression. CNVs were common in melanoma, with gain of *CDK4* or *CCND1* in 37% and 18% of cases respectively, and hemizygous or homozygous loss of *CDKN2A* in 56%. Three-quarters of all patients demonstrated a CNV in at least one of the three genes. The combination of *CCND1* gain with either a gain of *CDK4* and/or loss of *CDKN2A* was associated with poorer melanoma specific survival. In 47 melanoma cell lines homozygous loss, methylation or mutation of *CDKN2A* gene or loss of protein ($p16^{INK4A}$) predicted sensitivity to the CDK4/6 inhibitor PD0332991 while RB1 loss predicted resistance.

Significance

This study demonstrates that gene copy number variations in the CDK4 pathway are frequent events in primary invasive melanoma; with over 50% of patients exhibiting loss of *CDKN2A*, and that multiple CNVs in the CDK4 pathway are associated with worse clinical outcome. We further demonstrate that in melanoma cell lines loss of *CDKN2A* expression predicts sensitivity to the CDK4/6 inhibitor PD0332991 and RB1 loss predicts resistance. Taken together these findings lay the foundations for a clinical role for CDK4 inhibitors in treating melanoma, with the potential to use *CDKN2A*/p16^{INK4A} and RB1 expression to select patients likely to respond.

Keywords - Melanoma, *CDKN2A*, CDK4, p16^{INK4A}, RB1, Cyclin D1, PD0332991

Running Title - Targeting the CDK4 pathway in melanoma

Introduction

Over 197,000 cases of melanoma of the skin are diagnosed worldwide each year, resulting in 46,000 deaths annually (Ferlay et al., 2010). While survival rates for early-stage disease are good (80-90%), new effective therapies are required for patients whose cancers progress and/or become metastatic. *BRAF* and *NRAS* are the most frequently mutated oncogenes in melanoma, with rates of 45-55% and 15% respectively (Curtin et al., 2005; Devitt et al., 2011; Hacker et al., 2010; Houben et al., 2004; Long et al., 2011). The development and success of BRAF inhibitors in melanoma (Chapman et al., 2011; Flaherty et al., 2010) clearly indicates a prominent role of these oncogenes and oncogene addiction in this disease. We hypothesise that other oncogenic events are also critical to the development of melanoma and that potentially these events may induce dependence on individual oncogenes or key nodal points in pathways similar to *BRAF* or *NRAS*, which could create potential opportunities for targeted therapies. The

p16-cyclin D-CDK4/6-retinoblastoma protein pathway (CDK4 pathway) is likely one such pathway that melanomas may be reliant on, given the high frequency of genomic alterations in this pathway (Curtin et al., 2005; Walker et al., 1998) and that these events can transform melanocytes (Sheppard and McArthur, 2013).

Cyclin-dependent kinase 4 (CDK4) is a serine/threonine kinase that is a central regulator of the G1-S transition of the cell cycle. CDK4, or the highly homologous CDK6, when complexed with the D-type cyclins promotes cell cycle progression through the phosphorylation of several factors including the retinoblastoma protein. Subsequent phosphorylation of RB1 by the CDK2/CyclinE complex diminishes its ability to repress RNA polymerase I and III thus impacting on protein synthesis and in addition represses gene transcription through the E2F family of transcription factors that coordinate cell-cycle progression, nucleotide biosynthesis, DNA replication, mitotic progression, and DNA damage repair (Harbour et al., 1999; Lundberg and Weinberg, 1998; Sheppard and McArthur, 2013). Further regulation occurs through a negative feedback loop whereby CDK4 (or CDK6) inactivation of RB1 relieves RB1 mediated repression of cyclin-dependent kinase inhibitor 2A (*CDKN2A* gene; $p16^{INK4A}$ protein) (Li et al., 1994), which in turn leads to a reduction in CDK4/6 activity. This feedback loop effectively works as a natural brake on activation of this pathway. Loss of $p16^{INK4A}$ results in loss of the negative feedback control on this pathway leading to increased CDK4/6 activity that promotes cell cycle progression. It is also plausible that increasing activity of CDK4 by mutation or increased expression of its binding partner Cyclin D1 or CDK4 itself may overcome this negative feedback control and further activate the pathway. This process could potentially generate dependency on CDK4 for maintenance of cell proliferation.

The CDK4 pathway is known to be dysregulated in many cancers (Knudsen and Knudsen, 2008) and there is already strong evidence for a critical role of the CDK4 pathway in melanoma.

Sporadic human melanomas show high frequencies of genomic aberrations in *CDKN2A*, *CDK4*, and *CCND1* by gene gain, loss or mutation (Curtin et al., 2005; Walker et al., 1998). Inhibitory mutations in *CDKN2A* or activating mutations in *CDK4* in the germline of humans leads to a 50-fold increase in the risk of developing melanoma (Hussussian et al., 1994; Kamb et al., 1994; Zuo et al., 1996) and this predisposition to melanoma is reflected in mouse models with loss of *CDKN2A* or activation of CDK4 (Hacker et al., 2006; Krimpenfort et al., 2001; Rane et al., 2002; Sharpless et al., 2001; Sotillo et al., 2001). Taken together these data strongly indicate that melanocytes have a powerful predilection to be transformed by activation of the *CDK4* pathway, and raises the possibility of oncogenic addiction to this pathway that could be therapeutically targeted and exploited.

The prominent role of CDK4 pathway activation in cancer has led to the development of inhibitors of CDK4/6 suitable for clinical evaluation. PD0332991 is the most advanced in clinical development, with studies to date showing the compound to have favourable pharmacokinetics, absence of significant off-target toxicities (Fry et al., 2004) and importantly, evidence of target modulation *in vivo* in humans (Leonard et al., 2012). In this study we assessed gene copy number and protein levels for key members of the CDK4 pathway in a large clinical cohort of primary melanoma. In addition, and given the preclinical data indicating that melanoma formation and progression may be dependent on the CDK4 pathway we assessed the sensitivity of a panel of melanoma cells to the specific CDK4/6 inhibitor PD0332991 and correlated this with specific changes in this pathway. These studies are aimed at assessing PD0332991 as a potential novel therapeutic for the treatment of melanoma and identifying a potential target patient population for focussed clinical development of CDK4 inhibitors.

Results

Frequency of individual gene copy number variations of *CDK4*, *CCND1* and *CDKN2A* and the relationship to patient outcome and both *BRAF* and *NRAS* mutation status

Gene copy number variations (CNVs) for *CDK4*, *CCND1* and *CDKN2A* were evaluated via fluorescence in situ hybridization (FISH) assay (Table 1 and Figure 1). Gain of *CDK4* copy number was found in 37% (30/81) of cases, with an average gene copy number of 3.14 (range 2.40 - 6.76) for gain compared to 1.79 (range 1.50 - 2.10) for normal. Gain of *CCND1* was seen in 18% (22/121) of cases with an average gene copy number of 4.20 (range 2.42 - 9.66) for gain compared to 1.69 (range 1.38 - 2.27) for normal. Loss of *CDKN2A* was identified in 56% (63/113) of cases, with 50% (57/113) having hemizygous loss and 5% (6/113) having homozygous loss. The 57 cases with hemizygous loss had an average gene copy number of 0.80 (range 0.38-1.34) compared to 1.61 (range 1.32 - 2.06) for normal. Individual CNVs were more common in wild type than *NRAS* and *BRAF* mutated melanomas (Table 2). Gain of *CCND1* was rarely seen together with *BRAF* mutations (4/121) whereas *CDK4* gain (4/81) or *CDKN2A* loss (10/113) occurred infrequently together with *NRAS* mutations. We had complete follow up data for 131/143 patients, with median follow up time of 6 years and 6 months. Thirty-five patients had died, 27 due to melanoma. No significant interactions were found for individual gene CNVs and melanoma specific survival (Figure 2A-C). Mutations in *BRAF* or *NRAS* are known to confer poor survival in melanoma (Houben et al., 2004; Long et al., 2011). When patients were stratified by *BRAF* or *NRAS* mutation status, individual CNVs did not alter survival outcomes (data not shown). With regards to clinical and pathological variables known to be prognostic in melanoma, including Breslow thickness, ulceration, regression and mitotic rate, we found that individual CNVs in the CDK4 pathway did not further stratify patient survival (Table 1).

Frequency of multiple gene copy number variations and the relationship to patient outcome and both *BRAF* and *NRAS* mutation status

Sixty-eight cases were evaluated for all three genes (Table 3); 25% (17/68) showed no gene variations, 47% (32/68) showed a single gene variation, 24% (16/68) showed a double gene variation, and 4% (3/68) showed variations in all three genes. The combination of *CCND1* gain with *CDKN2A* loss was the most prominent double gene variation accounting for 56% (9/16) of all double gene variations (Table 3). When analysing combinations of gene CNVs with regards to patient outcome, we found that a gain of *CCND1* in combination with a gain in *CDK4* and/or loss of *CDKN2A* led to significantly poorer survival ($p<0.03$) (Figure 2D). No other combinations showed an association with patient survival. Our results suggest that single CNVs are more common in patients that are wild type for *BRAF* or *NRAS* (58% wild type; 35% *BRAF* mutation; 30% *NRAS* mutation), while multiple CNVs are more common in *BRAF* or *NRAS* mutant tumors (11% wild type; 40% *BRAF* mutation; 40% *NRAS* mutation; Table 3), although the number of patients in each group is relatively small.

Protein expression of CDK4, Cyclin D1 and $p16^{INK4A}$ in primary invasive melanoma

Results for protein expression for Cyclin D1 and $p16^{INK4A}$ were obtained for 130 and 132 cases respectively. We were unable to reliably optimise and validate immunohistochemistry for CDK4. For Cyclin D1 strong staining in 72% (94/130), moderate in 22% (29/130) and weak or no staining in 5% (7/130) of tumors was observed (Supplementary Figure 1). For $p16^{INK4A}$ we observed 38% (50/132) of cases with strong staining, 23% (30/132) with moderate staining and 39% (52/132) with weak or no staining (Supplementary Figure 1). There was no correlation between protein expression of Cyclin D1 with gene copy number of *CCND1*. Loss of $p16^{INK4A}$ expression by IHC was associated with homozygous gene loss of *CDKN2A* by FISH, and there was a trend of decreasing $p16^{INK4A}$ protein expression with decreasing *CDKN2A* gene copy number though this was not statistically significant. With regards to patient outcomes, protein

expression of Cyclin D1 or $p16^{INK4A}$ did not stratify patients, and expression was similar across wild type, *BRAF* and *NRAS* mutant tumors (data not shown).

Melanoma cell line sensitivity to PD0332991

The high frequency of gene alterations observed in the CDK4 pathway in primary melanoma led us to determine the ability of PD0332991 (Fry et al., 2004), a CDK4/6 specific inhibitor, to inhibit cell proliferation in a panel of 47 melanoma cell lines. Figure 3A shows the concentration of PD0332991 that inhibited cell proliferation by 50% (GI50), with GI50s ranging from 30nM to 9μM. The majority of cell lines (37/47) were sensitive to PD0332991 and there was a clear separation between cells that responded to PD0332991 (having a GI50 less than 1μM) and those cells that were resistant. Proliferation rates for a subset of melanoma cell lines was determined and this did not correlate with sensitivity to PD0332991 (Supplementary Figure 3). Examination of the images generated from the dose response curves indicated that PD0332991 was having a cytostatic effect rather than inducing cell death (data not shown), and cell cycle analysis on a subset of sensitive cells confirmed this, demonstrating that PD0332991 induced a G0/G1 cell cycle arrest and little if any cell death (Supplementary Figure 2).

Genomic predictors of PD0332991 sensitivity

To assess how changes in the CDK4 pathway may impact on sensitivity to PD0332991 we gathered information from COSMIC, Cancer Cell Line Encyclopedia and published papers (Barretina et al., 2012; Dutton-Regester et al., 2012; Forbes et al., 2011; Stark and Hayward, 2007) and, in addition, sequenced all wild type and mutant $p16^{INK4A}$ cell lines to confirm their status (Table 4). Loss of functional $p16^{INK4A}$ due to gene deletion (49% of cells), methylation (4% of cells) or mutation (19% of cells) was the most prevalent alteration occurring in 71% of cell lines and this significantly correlated with PD0332991 sensitivity (Fisher's exact test

p<0.02). Of the 4 cell lines that had a R24C activating mutation in CDK4, 3 of these were sensitive to PD0332991 indicating that this mutation may also confer sensitivity. Mutations in BRAF and NRAS were found in 70% and 13% of cells respectively, and did not correlate with PD0332991 sensitivity. Together these data demonstrate that activation of the CDK4 pathway either through loss of $p16^{INK4A}$ or mutation in CDK4 confers sensitivity to PD0332991, which is consistent with the concept that melanoma with these genetic alterations become dependent on the CDK4 pathway.

Western Analysis of Retinoblastoma 1 Protein and $p16^{INK4A}$

Previous studies in other cell types have indicated that loss of RB1 confers resistance to PD0332991 (Comstock et al., 2013; Dean et al., 2012; Fry et al., 2004; Konecny et al., 2011; Michaud et al., 2010). To determine if RB1 loss was conferring resistance to PD0332991 in melanoma cells we performed western analysis on all the resistant cells and a subset of sensitive cell lines (Figure 4). All sensitive cell lines had detectable RB1 protein. Two resistant cell lines, A2058 and C013 had undetectable protein, consistent with resistance to PD0332991 being due to loss of *RB1*. Loss of functional $p16^{INK4A}$ was associated with sensitivity to PD0332991, so we sought to determine if the cell lines that were both sensitive to PD0332991 and wild type for *CDKN2A* expressed $p16^{INK4A}$ protein (Figure 4). Sixteen sensitive cell lines including all cells that were wild type for $p16^{INK4A}$ were tested for $p16^{INK4A}$ protein expression. Of the seven cell lines that had no obvious *CDKN2A* gene deletion, methylation or mutation, all had detectable CDKN2A mRNA (data not shown) but only two had detectable protein (SK-MEL28 and C057). The loss of $p16^{INK4A}$ expression in cell lines with CDKN2A mRNA suggests that in some cell lines loss of $p16^{INK4A}$ occurs at either the translational or post-translational level. Of the two cell lines that did express $p16^{INK4A}$ these cell lines had an activating mutation in CDK4. In contrast to the sensitive cell lines, all six resistant cell lines that were wild type for p16

INK4A had detectable protein. These data further support the concept that loss of p16INK4A protein and activation of the CDK4 pathway confers sensitivity to PD0332991.

Discussion

In the present study we show that in a large cohort of primary invasive melanomas gene alterations of the CDK4 pathway are common. Activating events in two key drivers of the CDK4 pathway, CDK4 and Cyclin D1 and loss of the key inhibitor p16INK4A occurs in at least 75% of tumors, the most prominent being either hemizygous or homozygous loss of *CDKN2A* which occurs in 56% of tumors. These findings are in agreement with previous studies (Curtin et al., 2005) demonstrating amplifications of *CCND1* and *CDK4* are common and that loss of *CDKN2A* occurs in approximately 50% of cases. A gene gain in either *CCND1* or *CDK4* would likely increase signalling through the CDK4 pathway, and this signalling would be greatly amplified with the additional loss of the CDK4 inhibitor p16INK4A, potentially leading to a more aggressive disease state. This hypothesis is supported by the observation that patients with *CCND1* gain together with a loss of *CDKN2A* and/or a gain in *CDK4* had a significantly poorer survival rate. Our data suggest that this amplified CDK4 pathway signalling is a common early event in melanoma, is more common in tumors wild type for BRAF and NRAS, and that it may play a role in disease aggressiveness. Furthermore, it raises the possibility that in melanoma CDK4 pathway activation may provide an additional oncogenic addiction irrespective of BRAF and NRAS mutant status.

Given the prevalence of activating events in the CDK4 pathway in melanoma we assessed a melanoma cell line panel for sensitivity to the CDK4/6 inhibitor, PD0332991. We demonstrate that the majority of melanoma cell lines are sensitive to PD0332991. Consistent with the concept of CDK4 pathway dependent oncogene addiction, cell lines with loss of functional

p16^{INK4A} either through gene deletion, methylation or mutation were significantly more sensitive to PD0332991 than cells with wild type p16^{INK4A}. Of the seven PD0332991 sensitive cell lines that had wt-p16^{INK4A}, only two expressed detectable p16^{INK4A} protein. These two sensitive cell lines have a mutation in CDK4 which results in an inability of p16^{INK4A} to inhibit CDK4 action, thus these cells would still be reliant on CDK4/6 signalling. In complete contrast all six resistant cell lines with wt-p16^{INK4A} cells had detectable p16^{INK4A} protein, indicating that they are likely under chronic p16 mediated CDK4/6 inhibition. This chronic CDK4 inhibition likely renders them independent of this pathway for cell cycle progression and therefore resistant to CDK4/6 inhibition. These data suggest that p16^{INK4A} protein expression would be an excellent predictive response biomarker for PD0332991 in melanoma patients. Furthermore, this association between sensitivity to PD0332991 with loss of p16^{INK4A} expression has been previously reported for ovarian (Konecny et al., 2011), breast (Finn et al., 2009) and renal cell carcinoma cell lines (Logan et al., 2013) indicating that it is likely a robust biomarker of sensitivity across many cancer types.

Melanoma cells that were RB1 null were also resistant to PD0332991. In agreement with our study, previous *in vitro* and *in vivo* studies on cell lines derived from breast and ovarian cancer and in glioblastoma multiforme tumors have clearly defined loss of RB1 as a key determinant of resistance to PD0332991 (Comstock et al., 2013; Dean et al., 2012; Fry et al., 2004; Konecny et al., 2011; Michaud et al., 2010). With chronic loss of RB1 there is also loss of the negative feedback to p16^{INK4A}, thus causing a constant high level of p16^{INK4A}, as seen in our cell lines with RB1 loss (C013 and A2058; Figure 4). This high level of p16^{INK4A} would lead to chronic inhibition of CDK4/6 activity. Thus in RB1 null cells, through the high levels of p16^{INK4A} there is not only loss of CDK4/6 signalling to RB1 but also to other substrates involved in cell growth and proliferation therefore making it necessary for the cell to find other avenues to progress through the cell cycle (Sheppard and McArthur, 2013).

Our data have shown a high frequency of copy number variations involving key CDK4 pathway genes *CDK4*, *CCND1* and *CDKN2A* in primary invasive melanomas, indicating that these changes are an early event in melanoma. Similarly, our melanoma cell lines also demonstrated a high frequency of gene alterations in *CDKN2A* and *CCND1.* Furthermore, our PD0332991 response studies suggest that these changes do confer a state of dependence on the CDK4 pathway for cell cycle progression. As the melanoma progresses we predict that these events remain as potent drivers of CDK4 pathway activation and previous studies would indicate that at least the frequency of loss of $p16^{INK4A}$ is more prevalent in advanced stages of disease (Sanki et al., 2007). Thus the present study provides the first evidence towards the use of CDK4 inhibitors in treating melanoma. We have potentially identified *CDKN2A* gene status or $p16^{INK4A}$ protein expression as predictive biomarkers to select a sensitive patient population for efficacy of PD0332991 and conversely, RB1 loss, as a biomarker of resistance. In addition, the frequent activation of the CDK4 pathway across all melanomas irrespective of BRAF and NRAS mutation status and that these mutations do not alter sensitivity to the CDK4/6 inhibitor provides an opportunity for treating all melanoma patients with activation of the CDK4 pathway. Further work is required to elucidate the mechanisms of resistance to PD0332991 and to design rational combination therapeutic strategies that will improve outcomes for patients with primary invasive melanoma**.**

Methods

Patient cohort

The clinical and pathological characteristics for this patient cohort have been previously described (Handolias et al., 2010; Liu et al., 2007). Briefly, from May 2003 to September 2004, 251 consecutive patients with a new diagnosis of primary cutaneous melanoma were recruited from two melanoma referral centres in Melbourne (Peter MacCallum Cancer Centre and The Victorian Melanoma Service, Alfred Hospital). Study investigations were performed after

approval by the local Human Research Ethics Committees. Informed consent was obtained from all participants. Patient characteristics relevant to the present study are summarised in Table 1.

Tissue Micro Arrays

Formalin fixed paraffin embedded (FFPE) tumor tissue blocks were obtained for 180 patients. For each FFPE tumor block a section was cut and stained with Haematoxylin and Eosin for identification by a pathologist of areas of invasive melanoma. Of the initial 180 cases, 37 blocks were excluded as they showed no, or very little, invasive tumor component; 11 blocks were deemed to have insufficient invasive tumor area for inclusion on a tissue microarray (TMA); these cases had FISH and IHC performed on whole sections instead; 132 blocks had sufficient invasive tumor area for inclusion on TMAs. Up to four representative 1mm tumor cores were punched for each tumor block.

Fluorescence in situ hybridisation

FISH assays utilising FFPE tumor sections were optimised and validated for *CDK4*, *CCND1* and *CDKN2A*. Four micron sections were cut onto superfrost+ slides, then deparaffinised in xylene and rehydrated through graded alcohols to water. Target retrieval was performed in Heat Pretreatment Solution (Invitrogen) either in a pressure cooker at 125^{o}C for 2 minutes (*CCND1* and *CDKN2A*) or at 95^{0}C for 30 minutes *(CDK4*), followed by several washes in water. Sections were treated with Enzyme Pretreatment Reagent (Invitrogen) for 20 minutes at room temperature (*CCND1* and *CDKN2A*) or 30 minutes at 37C *(CDK4*). Slides were dehydrated through graded ethanol and air-dried. Probe (*CDK4*/SE12, Kreatech; *CCND1*/CEP11, Vysis; *CDKN2A*/CEP9, Vysis) was added to the sections, which were cover slipped and sealed with rubber cement to prevent evaporation during hybridisation. Slides were denatured for 5 minutes at 80^{o}C (*CCND1* and *CDKN2A*) or 20 minutes at 85C *(CDK4*) then hybridised for 17

hours at 37°C on a StatSpin Hybridiser (Dako). After hybridisation the coverslips were removed and slides were washed for 2 minutes in a 0.5x SSC stringent wash buffer at room temperature followed by 5 minutes in a 0.5x SSC stringent wash buffer at 75°C. After several washes in water, sections were dried and mounted in Vectashield Mounting Medium with Dapi (Vector) and coverslipped. Sections were stored at 4°C in the dark prior to scoring.

Scoring of FISH was performed on an Olympus BX51 fluorescence microscope. For each tumor core or section areas of tumor cells were identified at low magnification using the DAPI filter. Tumor areas were then scanned at high magnification (x100) to assess for heterogeneity of signal patterns. A minimum of 50 cells, typically 15-25 from each of two to three fields, representative of the total tumor area, were scored for the number of gene (red) and chromosome (green) signals using a 100x oil immersion lens. Raw data was entered into a Microsoft Excel spreadsheet for calculation of average values for gene and chromosome numbers and gene/chromosome ratio for each case. Cut off values were determined for gain of *CDK4* or *CCND1* (average gene copy >2.40) or loss of *CDKN2A* (hemizygous loss = average gene copy <1.40 or gene/chromosome ratio 0.11-0.80; homozygous loss = gene/chromosome ratio < 0.10).

Immunohistochemistry

Four-micron FFPE sections from the TMA blocks were used for immunohistochemistry (IHC). For $p16^{INK4A}$ (clone E6H4; Cintec) and Cyclin D1 (clone SP4;Ventana) the fully automated Ventana Benchmark Ultra (Ventana) was used. After dewaxing, antigen retrieval was achieved using high pH Ventana CC1 retrieval solution for 32 minutes followed by incubation with $p16^{INK4A}$ (antibody purchased pre-diluted) or Cyclin D1 antibody (1:100 dilution) for 32

minutes. Detection was achieved through Ultraview universal AP Red detection reagents, and slides were mounted using Pertex mounting medium.

A pathologist performed scoring of IHC staining. The scoring system developed involved scores of 0-3 for both intensity of staining and proportion of tumor cells stained. For intensity scores were 0= negative, 1 = weak, 2 = moderate, 3 = strong. For proportion, scores were 0 = negative, 1 = 1-10%, 2 = 11-50%, and 3 = >50%. For each core, the intensity and proportion scores were added together to give a score out of six. A final score of 0 = negative, 2.0 - 2.5 = weak, 3.0 - 4.5= moderate, and >4.5 = strong. For tumor samples with more than one core on the TMAs, the highest score across all cores was used.

Melanoma Cell Lines

Melanoma cell lines were obtained from ATCC, NCI, DSMZ and from the Australasian Biospecimen Network-Oncology Cell Line Bank at the QIMR (Supplementary Table 1). PCR based short tandem repeat (STR) analysis using 6 STR loci was performed on early passage cell lines. This analysis confirmed the individuality of each cell line and then was used routinely to confirm the identity of cells.

CDK4/6 inhibitor

PD0332991 (6-acetyl-8-cyclopentyl-5-methyl-2-((5-(piperazin-1-yl) pyridin-2-yl) amino) pyrido [2,3-d] pyrimidin-7 (8H) -one) a specific CDK4 and CDK6 inhibitor (Fry et al., 2004) was obtained from Pfizer Oncology.

Mutation and copy number data

Genomic DNA was extracted using QIAGEN DNeasy Blood and Tissue kit. Information on the genomic alterations in *CDKN2A, BRAF, NRAS, CDK4, CDK6* and *CCND1* for each cell line shown in Table 4 was obtained from published data sets (Barretina et al., 2012; Dutton-Regester et al., 2012; Forbes et al., 2011; Stark and Hayward, 2007) and by sequencing *CDKN2A* as previously described. (Lim et al., 2013).

Proliferation and Dose Response Assays

To assess the effects of PD0332991 on cell proliferation, cells were seeded in 96 well plates 48 hours prior to the addition of half-$\log_{10}$ doses of PD0332991 ranging from 1nM to 30μM or dimethylsulfoxide (DMSO) as vehicle control. Cell number was assessed using either the IncuCyte live cell imaging system (Essen Instruments) or using the standard sulforhodamine B (*SRB)* assay (Monks et al., 1991; Skehan et al., 1990). In all experiments cells were plated so that the cell confluency at the end of the incubation was less than 90% in control (DMSO) treated wells. GI50 for PD0332991 was defined as the concentration of drug that induced a 50% decrease in cell number and was determined using GraphPad Prism (GraphPad Software). For determination of doubling time for each cell line, cells were plated and cell number assessed every 4hrs – 8hrs using the IncuCyte. Experiments were performed at least twice for each cell line and the average is shown.

Immunoblotting

Exponentially growing cells were lysed in RIPA buffer (50mM Tris, 100mM NaCl, 2mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 50mmol/L sodium fluoride, 10mM sodium vanadate) plus phosphatase inhibitor cocktail (Roche). Protein concentrations were

determined by DC Protein assay (BioRad). Lysates were subjected to SDS-PAGE, transferred to PDVF, immunoblotted and proteins visualized by Western Lightening Plus Enhanced Chemiluminescence (PerkinElmer). Primary antibodies used were total RB (BD Pharmingen #554136), $p16^{INK4A}$ (Santa Cruz C-20 #sc-468) and actin (MP Biomedicals, C4), with horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies (BioRad #170-6516, #170-6515).

Statistical analysis

GraphPad Prism statistical software was used to calculate frequency of CNVs and for generation of Kaplan Meier plots for melanoma specific survival using a Log-rank (Mantel-Cox) test. Follow up was for an average of 6 years and 6 months. Melanoma specific survival was defined as death due to melanoma and excluded patients who died from other causes. Chi-square tests were used to assess associations between mutation status and sensitivity. Differences of $p<0.05$ were considered statistical significant.

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Potential Conflict of Interest

Pfizer supplied the therapeutics PD0332991. James Christensen and Sophia Randolph are employees at and shareholders in Pfizer. Grant. McArthur, Rick Pearson and Karen Sheppard received commercial research funding from Pfizer.

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Figure 1 - **Representative images of fluorescence in situ hybridisation. (A)** *CDK4* gain - arrows point to cells with increased number of gene copies (red signals). **(B)** *CCND1* gain - arrows point to cells with increased number of gene copies (red signals). **(C)** *CDKN2A* hemizygous loss - arrows point to cells with loss of one gene copy (ie. one red signal, two green signals). **(D)** *CDKN2A* homozygous loss - arrows point to cells with loss of both gene copies (ie. no red signals, two green signals. **(E)** Normal - arrows point to cells with a normal (disomy) signal pattern (ie two gene (red) and two chromosome (green) signals) All images x100 magnification.

Figure 2 - Kaplan Meier plots for melanoma specific survival. Individually *CDK4* gain **(A)**, *CCND1* gain **(B)**, and *CDKN2A* loss **(C)** did not correlate with melanoma specific survival,

however the combination of *CCND1* gain with *CDK4* gain and/or *CDKN2A* loss **(D)** showed significantly poorer survival (P<0.05).

Figure 3 - Sensitivity of Melanoma cells to PD0332991. Cell proliferation GI50 values were generated from PD0332991 dose response curves for a panel of melanoma cell lines. Each bar represents the average of at least two independent experiments.

Figure 4 - Immunoblot of total RB1 and $p16^{INK4A}$ expression in a subset of melanoma cell lines. All PD0332991 resistant cell lines together with cell lines that were sensitive and expressed wild type $p16^{INK4A}$ were assessed for RB1 and $p16^{INK4A}$ protein expression. For $p16^{INK4A}$ a short exposure (SE) and a long exposure (LE) are shown. * Represents melanoma cells that are wild type for the $p16^{INK4A}$ gene.

Supplementary Figure S1 - **Representative images of immunohistochemistry. (A and B)** $p16^{INK4A}$ IHC; **(C and D)** Cyclin D1 IHC. **(A)** and **(C)** show examples of weak expression of $p16^{INK4A}$ and Cyclin D1 respectively; **(B)** and **(D)** show examples of strong expression of $p16^{INK4A}$ and Cyclin D1 respectively.

Supplementary Figure 2- Cell cycle analysis. PD0332991 sensitive cell lines were treated with 1μM PD0332991 (PD991) for either 24hrs (A) or 5 days (B) and then both cell death and the cell cycle phase determined by flow cytometry.

Supplementary Figure 3- Proliferation rate and sensitivity to PD0332991. (A) Proliferation rate was determined for a subset of exponentially growing melanoma cell lines. Data shown are the average of at least two independent experiments. **(B)** Proliferation rate was plotted against the sensitivity to PD0332991. There was no significant correlation.

Table 1 - Patient and tumor characteristics according to individual gene copy number variations of *CDK4*, *CCND1* and *CDKN2A*.

Characteristic	**All**	***CDK4* gain**	***CCND1* gain**	***CDKN2A* loss**
Total number	143	30/81 (37%)	22/121 (18%)	63/113 (56%)
Sex				
Male	78 (55%)	20 (67%)	10 (45%)	34 (54%)
Female	65 (45%)	10 (33%)	12 (55%)	29 (46%)
Age (years)				
Mean	56	59	58	53
Range	19-89	20-89	27-89	20-89
Clinical Stage				
IA	46 (34%)	8 (31%)	4 (22%)	18 (32%)
IB	41 (30%)	10 (38%)	5 (28%)	15 (26%)
IIA	25 (19%)	3 (12%)	5 (28%)	13 (23%)
IIB	23 (17%)	5 (19%)	4 (22%)	11 (19%)
Breslow thickness (mm)				
≤1.0	48 (34%)	8 (29%)	4 (21%)	20 (33%)
1.1-2.0	41 (29%)	10 (36%)	5 (26%)	15 (25%)
2.1-4.0	30 (21%)	4 (14%)	6 (32%)	13 (22%)
>4.0	24 (17%)	6 (21%)	4 (21%)	12 (20%)
Ulceration				

Present	31 (22%)	7 (26%)	9 (45%)	13 (22%)
Absent	109 (78%)	20 (74%)	11 (55%)	46 (78%)

All values are number of cases and in brackets is the % of total cases in that category. Total numbers of patients in each category vary due to some data being unobtainable.

Table 2 – Frequency of individual gene copy number variations of *CDK4*, *CCND1* and *CDKN2A* and their relationship with *BRAF* and *NRAS* mutation status.

Individual CNVs	Number of cases with both CNV & mutation status	Wildtype	*BRAF* mutation	*NRAS* mutation
***CDK4* gain**	28	14 (50%)	10 (36%)	4 (14%)
***CCND1* gain**	22	13 (59%)	4 (18%)	5 (23%)
***CDKN2A* loss**	61	33 (54%)	18 (30%)	10 (16%)

All values are number of cases and in brackets is the % of the total number of cases with the mutation for each copy number variation (CNV).

Table 3 – Frequency of multiple gene copy number variations of *CDK4*, *CCND1* and *CDKN2A* and their relationship with *BRAF* and *NRAS* mutation status.

Number of Gene CNV's	**All** (n=68)	**Wildtype** (n=38)	***BRAF* mutation** (n=20)	***NRAS* mutation** (n=10)
no CNV	**17 (25%)**	**9 (24%)**	**5 (25%)**	**3 (30%)**
1 CNV	**32 (47%)**	**22 (58%)**	**7 (35%)**	**3 (30%)**
CDK4 gain	3 (4%)	3 (8%)	0 (0%)	0 (0%)
CCND1 gain	13 (19%)	9 (24%)	3 (15%)	1 (10%)
CDKN2A loss	16 (24%)	10 (26%)	4 (25%)	2 (20%)
2 CNVs	**16 (24%)**	**4 (11%)**	**8 (40%)**	**4 (40%)**
CDK4 + CCND1 gain	3 (4%)	1 (3%)	1 (5%)	1 (10%)
CDK4 gain + CDKN2A loss	4 (6%)	1 (3%)	2 (10%)	1 (10%)
CCND1 gain + CDKN2A loss	9 (13%)	2 (5%)	5 (25%)	2 (20%)
3 CNV	**3 (4%)**	**3 (8%)**	**0 (0%)**	**0 (0%)**

In bold are the total number of cases with 0, 1, 2 or 3 copy number variations (CNVs). Below each of these values is a breakdown of the number of cases with the particular gain and/or loss. Table includes only those cases where CNVs were evaluable for all three genes.

TABLE 4: Mutations in *CDKN2A*, *CDK4*, *BRAF* and *NRAS*

Cell line	PD991 (GI50:nM)	*CDKN2A* (CNV & Mutations)	CDK4 (Mutations)	BRAF (Mutations)	NRAS (Mutations)	Reference
C054	30	HD	WT	WT	Q61K	(Dutton-Regester et al., 2012) (KD-R)
SK-MEL24	35	HD	WT	V600E	WT	(Barretina et al., 2012; Forbes et al., 2011)
SK-MEL3	41	WT	WT	V600E	WT	(Barretina et al., 2012; Forbes et al., 2011), (IH)
MALME-3M	43	HD	WT	V600E	WT	(Barretina et al., 2012)
HS294T	55	HD		V600E	WT	(Barretina et al., 2012)
C088	62	WT	WT	V600K	WT	(Dutton-Regester et al., 2012), (IH) (KD-R)
G361	65	HD	WT	V600E	WT	(Barretina et al., 2012; Forbes et al., 2011)
C052	79	Mutated (A20P:loss CDK4/6 binding,LOF)	WT	WT	WT	(Dutton-Regester et al., 2012), (IH) (KD-R)
MEL-Ho	88	HD	WT	V600E	WT	(Barretina et al., 2012; Forbes et al., 2011)
SK-MEL5	90	HD	WT	V600E	WT	(Barretina et al., 2012; Dutton-Regester et al., 2012; Forbes et al., 2011; Stark and Hayward, 2007)
A15	96	Methylated	WT	V600K	WT	(Dutton-Regester et al., 2012)
C002	96	HD	WT	WT	Q61K	(Dutton-Regester et al., 2012) (KD-R)
D35	101	Mutated (8bp deletion: truncation,LOF)	WT	WT	WT	(Dutton-Regester et al., 2012; Stark and Hayward, 2007)
C057	104	WT	R24C	V600E	WT	(Dutton-Regester et al., 2012), (IH) (KD-R)
C001	104	WT	WT	WT	Q61K	(Dutton-Regester et al., 2012), (IH) (KD-R)
D36	109	Mutated (R80Stop,LOF)	WT	V600E	WT	(Dutton-Regester et al., 2012; Stark and Hayward, 2007), (IH)
RVH-421	114	HD		V600E	WT	(Barretina et al., 2012; Forbes et al., 2011)
WM115	120	HD	WT	V600D	WT	(Barretina et al., 2012; Forbes et al., 2011)
COLO-800	129	HD	R24C	V600E	WT	(Barretina et al., 2012; Forbes et al., 2011)
C027	134	WT	WT	WT	Q61L	(Dutton-Regester et al., 2012), (IH) (KD-R)
D08	142	HD	WT	WT	Q61L	(Dutton-Regester et al., 2012; Stark and Hayward, 2007)
HT144	145	HD	WT	V600E	WT	(Barretina et al., 2012; Dutton-Regester et al., 2012; Forbes et al., 2011)

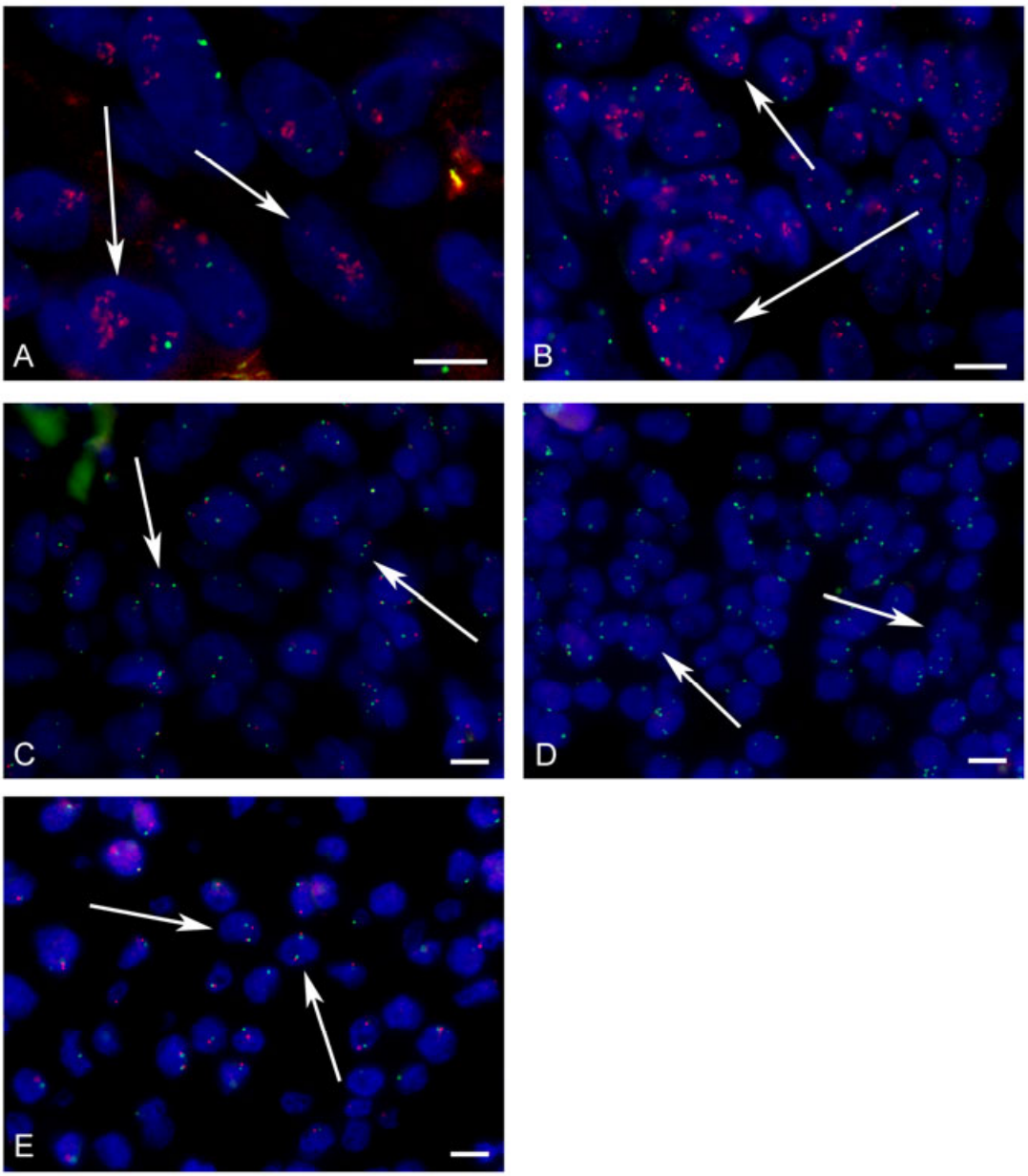
C062	150	HD	WT	V600E	WT	(Dutton-Regester et al., 2012) (KD-R)
D05	161	Mutated (Frame Shift:truncation,LOF)	WT	V600E	WT	(Dutton-Regester et al., 2012; Stark and Hayward, 2007)
C074	162	HD	WT	V600E	WT	(Dutton-Regester et al., 2012), (IH) (KD-R)
D17	164	Methylated	WT	V600E	WT	(Dutton-Regester et al., 2012; Stark and Hayward, 2007)
C021	167	WT	WT	WT	WT	(Dutton-Regester et al., 2012), (IH) (KD-R)
SK-MEL28	190	WT	R24C	V600E	WT	(Barretina et al., 2012; Dutton-Regester et al., 2012; Forbes et al., 2011; Stark and Hayward, 2007), (IH)
A375	192	Mutated (E61Stop,LOF)	WT	V600E	WT	(Barretina et al., 2012; Forbes et al., 2011), (IH)
CHL1	192	Mutated (W110Stop,LOF)	WT	WT	WT	(Barretina et al., 2012; Forbes et al., 2011)
LOX-IMVI	214	HD	WT	V600E	WT	(Barretina et al., 2012; Forbes et al., 2011)
C32	282	Mutated (157S-Nonstop extension)	WT	V600E	WT	(Dutton-Regester et al., 2012; Forbes et al., 2011; Stark and Hayward, 2007)
D04	301	HD	WT	WT	Q61L	(Dutton-Regester et al., 2012; Stark and Hayward, 2007)
WM266-4	305	HD	WT	V600D	WT	(Barretina et al., 2012)
A11	388	HD	WT	WT	WT	(Dutton-Regester et al., 2012)
C055	400	HD	WT	V600E	WT	(Dutton-Regester et al., 2012) (KD-R)
C089	586	HD	WT	V600E	WT	(Dutton-Regester et al., 2012) (KD-R)
C044	**1474**	**WT**	**WT**	**V600E**	**WT**	(Dutton-Regester et al., 2012), (IH) (KD-R)
MeWo	**1491**	**Mutated (R80Stop, LOF)**	**WT**	**WT**	**WT**	(Barretina et al., 2012; Forbes et al., 2011), (IH)
A02	**1867**	**WT**	**WT**	**V600E**	**WT**	(Dutton-Regester et al., 2012; Stark and Hayward, 2007), (IH)
D14	**2080**	**Mutated (P114L: loss CDK4/6 binding,LOF)**	**WT**	**V600E**	**WT**	(Dutton-Regester et al., 2012; Stark and Hayward, 2007), (IH)
D41	**2290**	**WT**	**WT**	**V600E**	**WT**	(Dutton-Regester et al., 2012; Stark and Hayward, 2007), (IH)
D20	**4558**	**WT**	**WT**	**V600E**	**WT**	(Dutton-Regester et al., 2012; Stark and Hayward, 2007), (IH)
A2058	**5637**	**WT**	**WT**	**V600E**	**WT**	(Barretina et al., 2012; Dutton-Regester et al., 2012; Forbes et al., 2011), (IH)
C071	**5693**	HD	**R24C**	**V600E**	**WT**	(Dutton-Regester et al., 2012) (KD-R)
C013	**7500**	**WT**	**WT**	**WT**	**Q61L**	(Dutton-Regester et al., 2012), (IH) (KD-R)

D24	**8923**	HD	**WT**	**WT**	**WT**	(Dutton-Regester et al., 2012; Stark and Hayward, 2007)

1. Mutations are highlighted in grey and bolded text are the resistant cell lines

2. Abbreviations: WT, wild type. HD, Homozygous Deletion. LOF, Loss-of-function. IH, in house assessment of CDKN2A mutation status.

3. KD-R, data available from Ken Dutton-Regester PhD Thesis (http://eprints.qut.edu.au/53305/1/Ken_Dutton-Regester_Thesis_Final.pdf)



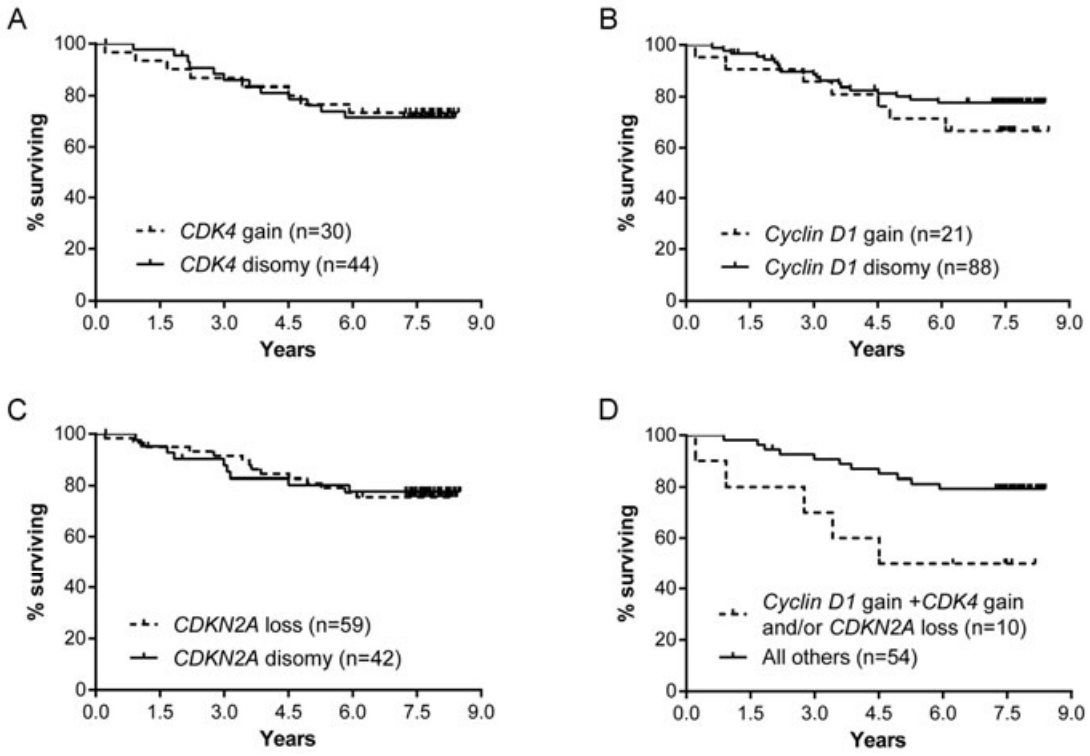


Figure 3 Young et al

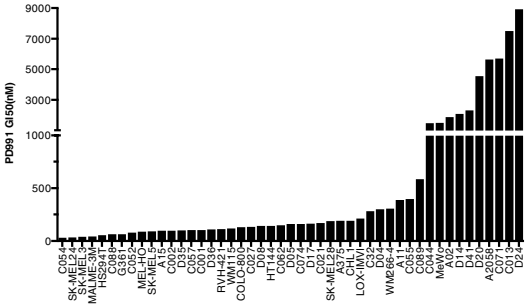
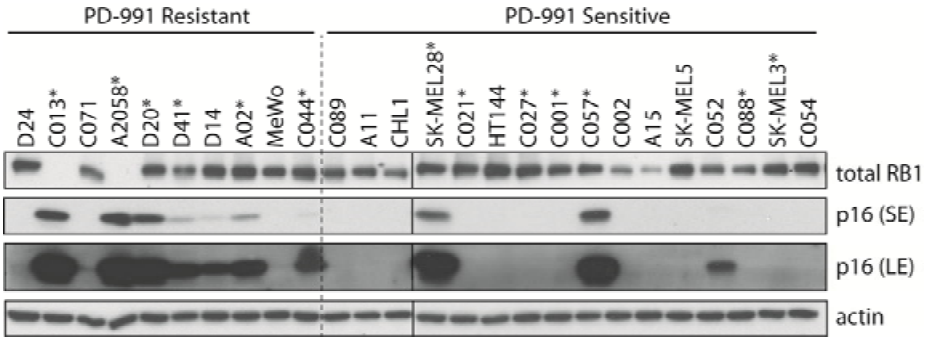


Figure 4 Young et al



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