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**Title:**

**Dual specificity protein phosphatase DUSP4 regulates response to MEK inhibition in *BRAF* wild-type melanoma**

**Running Title:**

**DUSP4 and MEK inhibition in *BRAF* wild-type melanoma**

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## Abstract

**Background:** Aiming to improve treatment options for *BRAF* wild-type melanoma, we previously conducted the DOC-MEK study of docetaxel with MEK inhibitor (MEKi) selumetinib or placebo, revealing trends to prolongation of progression free survival (hazard ratio 0.75,  $P=0.130$ ), and improved response rates (32% vs 14%,  $P=0.059$ ) with docetaxel plus selumetinib. *NRAS* status did not associate with outcome. Here, the aim was to identify novel biomarkers of response to MEKi.

**Methods:** A MEK 6-gene signature was quantified using NanoString and correlated with clinical outcomes. Two components of the gene signature were investigated by gene silencing in *BRAF/NRAS* wild-type melanoma cells.

**Results:** In melanomas of patients on the selumetinib but not the placebo arm, two gene signature components, dual specificity protein phosphatase 4 (DUSP4) and ETS translocation variant 4 (ETV4) were expressed more highly in responders than non-responders. *In vitro*, ETV4 depletion inhibited cell survival but did not influence sensitivity to MEKi selumetinib or trametinib. In contrast, DUSP4-depleted cells showed enhanced cell survival and increased resistance to both selumetinib and trametinib.

**Conclusions:** ETV4 and DUSP4 associated with clinical response to docetaxel plus selumetinib. DUSP4 depletion induced MEKi resistance, suggesting that DUSP4 is not only a biomarker but also a mediator of MEKi sensitivity.

**Clinical Trial Registration:** DOC-MEK (EudraCT no: 2009-018153-23)

## Background

The incidence of melanoma is increasing: it is now the fifth most common cancer in the UK and the second commonest cancer in adults aged 25-49 (<http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/skin-cancer/>). Early detection and treatment with surgical excision is often curative, but ~25% of patients develop local recurrences and/or metastatic disease <sup>1</sup>. The prognosis with advanced melanoma is poor, although advances in both immunotherapy and targeted therapy have had a significant impact on overall survival (OS), with median OS up to 24 months in some clinical trials <sup>2</sup>. Approximately 40-50% of melanomas harbour *BRAF* mutations that activate the RAS-RAF-MEK-ERK pathway, also known as the Mitogen Activated Protein Kinase (MAPK) pathway <sup>3</sup>. The most common *BRAF* mutation is a missense mutation leading to substitution of valine by glutamic acid at position 600 of the BRAF protein (V600E) <sup>4,5</sup>. Patients whose melanomas contain these mutations can be treated with mutant BRAF inhibitors such as vemurafenib or dabrafenib. These drugs achieve ~50% objective response rate (ORR) as monotherapy <sup>6,7</sup>, and up to 69% ORR when combined with MEK inhibitors (MEKi), which act downstream of BRAF to inhibit the activity of MEK1/2 <sup>8-10</sup>. However, treatment resistance and disease progression generally develop within 6-7 months on BRAF inhibitor (BRAFi) monotherapy and 11 months with BRAFi/MEKi combination treatment. For melanoma patients without *BRAF* mutations, targeted treatments have shown no significant benefit <sup>11,12</sup>.

We previously reported the outcomes of the multicentre Phase II DOC-MEK trial for patients with advanced *BRAF* wild-type melanoma <sup>13</sup>. Patients were randomised to

treatment with docetaxel plus either MEK1/2 inhibitor selumetinib (AZD6244, ARRY-142886) or placebo. The rationales for this combination were, firstly, that selumetinib has demonstrated preclinical efficacy in both *BRAF* wild-type and mutant melanoma models <sup>14</sup>. Secondly, resistance to taxane-induced apoptosis can be mediated by MAPK pathway activation, and thus concurrent MEK1/2 inhibition may potentiate the efficacy of taxane chemotherapy. Thirdly, the combination of selumetinib and docetaxel has been tested in melanoma and colorectal cancer xenografts <sup>15,16</sup> and in a Phase I trial <sup>17</sup>, with evidence of activity in a Phase II study in patients with non-small-cell lung cancer (NSCLC) <sup>18</sup>. In the DOC-MEK trial, patients treated with docetaxel plus selumetinib had an ORR of 32% compared with 14% in the docetaxel plus placebo arm ( $P=0.059$ ) <sup>13</sup>. There was a non-significant difference in progression-free survival (PFS) and *NRAS* mutation was not predictive of response to MEK inhibition <sup>13</sup>.

Here, we extend analysis of the DOC-MEK study with reference to a MEK functional activity gene signature, developed as a prognostic and/or predictive biomarker of MEK functional activation across a variety of tumour types <sup>19,20</sup>. We confirm no evidence of a predictive role for *NRAS* mutation status, but demonstrate that there is a correlation between response to treatment with docetaxel plus selumetinib and the MEK 6 gene signature score. Notably, two components of the signature, Dual specificity protein phosphatase 4 (DUSP4, also known as MKP2) and ETS translocation variant 4 (ETV4), correlate with partial response (PR) or complete response (CR) to docetaxel plus selumetinib but not to docetaxel plus placebo. This suggests that these proteins could act as biomarkers to identify patients likely to respond to the combination treatment. We investigate a possible role for DUSP4 and

ETV4 in mediating this response by assessing the effect of their depletion on the sensitivity of *BRAF* wild-type melanoma cells to MEK inhibition. We show that DUSP4 protein expression is suppressed by MEK inhibition, confirming its status as an ERK-regulated gene <sup>21</sup>. Furthermore, we demonstrate that DUSP4 depletion influences response to two MEK inhibitors, selumetinib and trametinib. Thus, DUSP4 expression is not only a potential biomarker for patient response to MEK inhibition, but is also a mediator of MEK inhibitor sensitivity.

## Methods

### ***Tumour mutation analysis by Next Generation Sequencing (NGS)***

Archival formalin fixed paraffin embedded (FFPE) tissue blocks were sectioned and enriched for tumour tissue by macro-dissection. Genomic DNA was extracted using the QIAamp FFPE Tissue kit and amplified using the Ion Ampliseq™ Library Kit 2.0. DNA sequencing was performed by the IonTorrent Personal Genome Machine (LifeTechnologies, Carlsbad, CA). A targeted cancer hotspot panel (designed using the Ion Ampliseq™ Cancer Primer Pool) was used to detect mutations in 46 known cancer-related genes (supplementary Table 1) <sup>22</sup>. The sensitivity of this assay is 5-10% (% of mutant DNA detectable in a background of wildtype DNA). When DNA was of insufficient quality for NGS, pyrosequencing was used to test for mutations in codons 12, 13 and 61 of *NRAS*.

### ***Gene expression analysis***

Tumour FFPE tissue was macro-dissected from 1-2 x 5 µm sections, RNA extracted using the RNeasy FFPE kit according to the manufacturer's instructions, and 100 ng of each RNA was analysed using the NanoString nCounter gene expression system <sup>23</sup>. The codeset was designed by NanoString Inc (Seattle, WA). Transcript counts were normalised between MEK signature genes and reference genes and transformed using the NanoString Normalisation Tool v2 (AstraZeneca Oncology Bioinformatics <http://CRAN.R-project.org/package=NAPPA>) in order to generate signature scores <sup>20</sup>. Signature scores were calculated blind to clinical outcomes.

### ***Cell lines and reagents***

CHL-1 cells were from the American Type Culture Collection (ATCC), and SK-mel-23 cells from Professor V. Cerundolo, Weatherall Institute of Molecular Medicine, University of Oxford. Cultures were maintained in Dulbecco's Modified Eagle's Medium with 10% foetal calf serum and 1% penicillin/streptomycin, in a humidified atmosphere of 10% CO<sub>2</sub>. Both cell lines were negative for mycoplasma (MycoAlert kit, Lonza Rockland Inc, Rockland US), and were authenticated by STR genotyping (Eurofins Medigenomix Forensik GmbH). Selumetinib and trametinib (Selleck) were stored as 10mM solutions in DMSO at -80°C.

### ***Western blotting and cell survival assays***

Cells were incubated with drug for 60-150 min before harvesting for Western blotting as previously described <sup>24</sup>, using antibodies to DUSP4 (#5149, Cell Signaling Technology (CST)), phospho-T202/Y204 ERK 1/2 (#4377, CST), total ERK 1/2 (#4695, CST),  $\beta$ -tubulin (T4026, Sigma), and actin (A3854, Sigma). For clonogenic survival assays drugs or vehicle control were added 24 hours after seeding and cells incubated in the presence of drug for 7 to 14 days.

### ***Gene silencing by siRNA transfection***

Cells were reverse transfected with 50 nM gene-specific or non-silencing Allstars (Qiagen) siRNA on day 1 using Dharmafect 1 (ThermoFisher), forward transfected with 50 nM siRNA on day 2, and re-seeded on Day 3 for clonogenic assays. DUSP4 was depleted using siDUSP4\_1 (#4392420-s4372, Ambion) and siDUSP4\_2 (J-003963-09, Dharmacon) and ETV4 using siETVA\_1 and \_2 (#106636 and #106637, Thermofisher).



## **Quantitative real-time PCR (qRT-PCR)**

RNAs were extracted using the Reliaprep™ RNA miniprep kit (Promega) and reverse-transcribed to complementary DNA (cDNA) using Superscript III First-Strand Synthesis Supermix (ThermoFisher). PCRs were performed on a 7500 Fast RT-PCR System (Applied Biosystems) using SYBR Green PCR mastermix (ThermoFisher) with the following primers: DUSP4 forward, 5'-GGGGTCCTGTGGAGATCCTT-3' and reverse, 5'-GGCAGTCCGAGGAGACATTC-3'; ETV4 forward 5'-GAGCGGAGGATGAAAGCCG-3' and reverse 5'-CCCATTTCGGGCGATTTG-3'; TUBA6 (housekeeping gene) forward 5'-CCCCTTCAAGTTCTAGTCATGC-3' and reverse 5'-ATTGCCAATCTGGACACCA-3'.

## **Statistical analysis**

The Kaplan-Meier method was used to obtain PFS and OS estimates by mutation status. As reported previously, the impact of *NRAS* mutation status was assessed by adding an interaction term with treatment in the Cox model, adjusting for stratification variables and using a significance level of 0.05<sup>13</sup>. Correlations between tumour mutation results, gene signature scores and clinical outcomes were analysed using Microsoft Excel, with significance determined using *t*-tests with a one-tailed distribution. *NRAS* VAF was compared with gene signature scores using Pearson's correlation coefficient. Western blot and cell survival data were analysed using GraphPad Prism v5, using *t*-tests to compare 2 groups and ANOVA for multiple groups in each case with a two-tailed distribution.

## Results

### ***Response to MEK inhibition does not correlate with NRAS mutation status***

Results from the DOC-MEK Phase II trial in *BRAF* wild-type advanced melanoma patients<sup>13</sup> demonstrated that docetaxel plus selumetinib did not significantly increase PFS or OS compared with docetaxel plus placebo, but there was a trend towards increased ORR in the selumetinib group ( $P=0.059$ ). There was no correlation between patient response and *NRAS* mutation status. Here, we extended this analysis by investigating the mutational status of 45 additional genes in a 46 gene cancer panel. Mutation status was determined for 64 of the 83 patients randomised in the DOC-MEK study, in 59 cases by NGS using targeted sequencing of 46 cancer-associated genes (Figure 1A, Supplementary Table 1) and in 5 cases (DM005, DM026, DM029, DM037, and DM083) using pyrosequencing. In total, 84 mutations were found in 49 of the 59 cases analysed using NGS, with 2 or more concurrent mutations found in 24 cases. The most commonly mutated gene was *NRAS* (45% of all mutations detected), followed by *TP53* (7%; Figure 1A, Supplementary Table 2). These findings are consistent with an analysis of 699 unselected (*i.e.* *BRAF* wild-type and mutated) melanomas using the same 46 gene cancer panel, where, after *BRAF* mutations (41% of cases), *NRAS* (22%) and *TP53* (17%) were the next commonest mutations<sup>22</sup>. In our *BRAF* wild-type population, four cases tested by NGS had a *BRAF* mutation which had not been detected during initial screening by hotspot mutation testing (Figure 1A, Supplementary Table 2). One of these was *BRAF* V600E, detected at a very low variant allele frequency (VAF) of 5.5%. Three cases harboured non-V600 *BRAF* mutations: K601E, G466R and N581S, rare mutations reported in the COSMIC database

(<http://cancer.sanger.ac.uk>). Non-V600 *BRAF* mutations, including K601E, have been associated with sensitivity to MEK inhibition <sup>25,26</sup>. The G466R and N581S *BRAF* mutations were found in melanomas that also harboured mutant *NRAS*. As activating *BRAF* and *NRAS* mutations are considered mutually exclusive <sup>4</sup>, these two *BRAF* mutations are probably non-activating, or may be low activity *BRAF* mutants that require upstream RAS activation. The patient with low VAF *BRAF* V600E (case DM067, Supplementary Table 2) was randomised to docetaxel plus placebo and progressed within 3.3 months of treatment. Those with non-V600 *BRAF* mutations were all randomised to docetaxel plus selumetinib. The patients with *BRAF* K601E (case DM072) and *BRAF* G466R + *NRAS* Q61H mutant melanoma (case DM071) progressed after 4 and 7 months respectively, each with stable disease as best response. The patient with *BRAF* N581S melanoma (case DM077) was found to have two concurrent *NRAS* mutations (Q61K, Q61R) and initially demonstrated a partial response to treatment, but again progressed quickly after 4 months. The most common concomitant mutations were *NRAS* and *TP53* (Supplementary Table 2). There was no apparent association between number of concomitant mutations per tumour and median PFS or OS (Supplementary Table 3).

### ***Relationship between NRAS mutation status and MEK 6 gene expression signature***

Forty-eight tumours from the DOC-MEK study were available for transcriptional analysis, using the NanoString platform to quantify the MEK 6 gene score. Supplementary Table 4 summarises these scores, the *NRAS* mutation data and DOC-MEK clinical outcomes. We tested for associations between the MEK 6 gene score and *NRAS* status, best overall response and derived benefit. There was a

higher mean MEK 6 gene score in *NRAS* mutant melanomas compared with *NRAS* wild-type melanomas ( $P=0.023$ , Figure 1B), but the differences were small and there was considerable overlap between the two groups. Since levels of MAPK activation may be significantly different between tumours with low vs high mutant *NRAS* VAF, we assessed the correlation between *NRAS* VAF where available and MEK 6 gene score and found a modest positive correlation (Pearson's correlation coefficient,  $r=0.51$ , Figure 1C).

### ***Response to MEK inhibition correlates with MEK 6 gene expression score***

To assess correlations between patient response and MEK 6 gene expression score, patients in the two arms of the trial were analysed separately by comparing those achieving CR/PR with patients having stable disease (SD) or progressive disease (PD) at first assessment (Figures 2A, 2B). For patients treated with docetaxel plus placebo there was no significant difference in MEK 6 gene score between responders and those with SD/PD at first assessment (mean scores  $9.28 \pm 0.16$  and  $9.56 \pm 0.15$  respectively,  $P=0.222$ , Figure 2A). In contrast, patients achieving CR or PR on docetaxel plus selumetinib had a higher MEK 6 gene score than those with SD/PD at first assessment (mean scores  $10.14 \pm 0.17$  and  $9.34 \pm 0.31$  respectively,  $P=0.026$ , Figure 2B). However, there was again considerable overlap between the two populations. The absolute difference between mean scores was small, and as there were only 4 responding patients in the docetaxel plus placebo arm, these results should be interpreted with caution.

Since patients with prolonged disease stabilisation can also be considered to have derived benefit from treatment, a second analysis was performed. Gene expression

scores were compared between those who “derived benefit”, i.e. had CR, PR or SD  $\geq 6$  months, and those with “no derived benefit”, i.e. PD at first assessment or SD  $< 6$  months (Figures 2C, 2D). As previously, for patients treated with docetaxel plus placebo there was no difference in the MEK 6 gene score between those who derived benefit and those who did not (mean values of  $9.21 \pm 0.18$  and  $9.64 \pm 0.17$  respectively,  $P=0.094$ , Figure 2C). With more patients in the ‘benefit’ group, this analysis may be considered more reliable. Patients treated with docetaxel plus selumetinib who were considered to have derived benefit again had a higher MEK 6 gene score than those who did not benefit (mean scores  $9.93 \pm 0.16$  and  $9.11 \pm 0.58$  respectively,  $P=0.038$ , Figure 2D), with a similar pattern of distribution as in Figure 2B. Thus, using either approach to categorise clinical benefit, this analysis showed a higher mean MEK 6 gene score in the melanomas of patients responding to docetaxel plus selumetinib, but not to docetaxel plus placebo. However, the absolute difference in mean score using either approach was small.

In a third approach, we analysed expression of individual components of the MEK 6 gene signature, comparing those who responded (CR plus PR) with those with PD at first assessment. Gene expression data were available for 8/13 responders and 2/5 experiencing early progression in the combination treatment arm, and for 3/6 responders and 11/20 experiencing early PD in the docetaxel plus placebo arm. Whilst the numbers are small, the MEK 6 gene score data revealed potentially interesting differences in the expression of DUSP4 and ETV4, which showed significant differences in mean values ( $p < 0.05$ ) between the responders and non-responders in the selumetinib group (Table 1), but not in the placebo group (Supplementary Table 5). Thus, patients who responded to treatment had greater

expression of both DUSP4 and ETV4 compared with those experiencing early PD, and these differences were found only in the docetaxel plus selumetinib group.

Since higher expression of DUSP4 and ETV4 mRNA was associated with clinical response to MEK inhibition, we hypothesised that depletion of each of these proteins might have the reverse effect and induce resistance to MEK inhibition. We therefore decided to test this *in vitro* using *BRAF* wild-type melanoma cell lines CHL-1 and SK-MEL-23, and two MEK inhibitors, namely selumetinib, the MEK inhibitor used in the DOC-MEK clinical trial, and trametinib, the first MEK inhibitor approved by the FDA for use in clinical practice (<https://www.cancer.gov/about-cancer/treatment/drugs/fda-trametinib>). The aim of using two inhibitors was to check whether any observed changes were likely to be class effects, rather than specific to selumetinib. Western blots for phospho-T202/Y204 ERK 1/2 levels were used as a readout for MEK activity.

### ***MEK inhibition downregulates DUSP4 in BRAF wild-type melanoma cells***

We first assessed endogenous expression of DUSP4 and ETV4, which were detectable at the protein and mRNA level in both cell lines (Figures 3A, 3B), although CHL-1 showed considerably lower levels of expression of both proteins than SK-MEL-23. We also tested the effect of MEK inhibition on DUSP4 expression, using both selumetinib and trametinib. Selumetinib inhibited ERK phosphorylation at  $\geq 100\text{nM}$ , whereas trametinib caused inhibition at lower concentrations ( $\geq 3\text{nM}$ ) in both cell lines (Figure 3C, Supplementary Figures 1A, 1B), in keeping with the known  $\text{IC}_{50}$  values for each drug<sup>14,27</sup>. In both cell lines, DUSP4 expression also decreased as inhibitor concentration increased (Figure 3C, Supplementary Figures 1A, 1B),

consistent with reported control of its transcription by phosphorylated ERK1<sup>21</sup>.  
Trametinib suppressed ERK phosphorylation for 24 hours, with partial return of  
signal at 48-72 hours, while inhibition of DUSP4 expression persisted for at least 72  
hours (Supplementary Figure 1C).

### ***ETV4 depletion does not alter sensitivity of BRAF wild-type melanoma cells to MEK inhibition***

We assessed the influence of ETV4 on response to MEK inhibition, by depleting  
ETV4 and measuring cell survival and MEK inhibitor SF<sub>50</sub> values (drug concentration  
suppressing survival to 50% of control values) in clonogenic survival assays. ETV4  
knockdown was very effective in CHL-1 cells, with residual ETV4 mRNA of  $1.16 \pm$   
 $0.53\%$  and  $4.14 \pm 0.65\%$  for siETV4\_1 and siETV4\_2 respectively compared to the  
Allstars control siRNA transfectants (Supplementary Figure 2A, left). For SK-MEL-23  
cells the equivalent values were  $18.02 \pm 13.02\%$  and  $4.28 \pm 1.84\%$  (Supplementary  
Figure 2A, right). ETV4 depletion had a largely detrimental effect on cell survival in  
SK-MEL-23 but not in CHL-1 cells (Supplementary Figure 2B). Supplementary  
Figures 2C and 2D show results of representative survival assays testing effects of  
ETV4 depletion on response to trametinib, and summarise SF<sub>50</sub> values from 3  
independent assays in each cell line. We found no evidence that ETV4 depletion  
influenced the response of either cell line to MEK inhibition by trametinib  
(Supplementary Figures 2C, 2D). However, the presence of relatively few surviving  
colonies in ETV4 depleted cultures, especially of SK-MEL-23 (Supplementary Figure  
2B, right), could have contributed to the variation in trametinib SF<sub>50</sub> data  
(Supplementary Figure 2D), so we cannot exclude a small effect on trametinib  
response.

***DUSP4 depletion induces resistance of BRAF wild-type melanoma cells to MEK inhibition***

Next, we used siRNAs to deplete DUSP4 and measured cell survival and MEK inhibitor SF<sub>50</sub> values. Both DUSP4 siRNAs used induced effective DUSP4 depletion, at both the mRNA and protein level (Figure 4A, 4B). We tested the duration of DUSP4 knockdown in CHL-1 cells and demonstrated that depletion lasted at least 7 days (Figure 4C). Compared with controls, DUSP4 depleted cells showed an increase in cell survival that was significant in CHL-1 cells (\*\*P=0.0187 and 0.0154 for siDUSP4\_1 and \_2 respectively, Figure 4D left), but not in SK-MEL-23 cells (Figure 4D right).

Finally, we tested the sensitivity of DUSP4 depleted cells to both MEK inhibitors. The results are shown in Figure 4E, and the data are summarised in Table 2. In both cell lines with both MEK inhibitors there was a consistent shift to the right of the MEK inhibitor dose-response curve (Figure 4E), with 2.5 – 6.8 fold increase in SF<sub>50</sub> values in DUSP4 depleted cells compared with controls (Table 2). These results indicate that DUSP4 depleted cells were less sensitive to both selumetinib and trametinib than the control transfectants. Thus DUSP4 protein levels are not only affected by MEK inhibition but also alter cellular response to this class of drug.



## Discussion

There are fewer options for treating *BRAF* wild-type advanced melanoma than for the *BRAF* mutated population. DOC-MEK was the first published randomized trial in a selected *BRAF* wild-type melanoma population, and indicated that a proportion of such patients could benefit from combined treatment with docetaxel and selumetinib<sup>13</sup>. During the conduct of the DOC-MEK study, MEK inhibitor binimetinib (MEK162) was reported to have a 20% ORR (6/30) in patients with *NRAS* mutated melanoma in a monotherapy Phase II study<sup>28</sup>. However, in a retrospective analysis of DOC-MEK data, we observed no correlation between *NRAS* status and clinical outcome in either treatment arm<sup>13</sup>, reflecting previously published data with selumetinib alone<sup>29</sup> and selumetinib plus dacarbazine/docetaxel chemotherapy<sup>30</sup>.

We extended the mutational analysis to a 46-gene cancer panel and found no correlation between number of concomitant mutations and clinical outcome. We therefore investigated the hypothesis that the expression of genes that correlate with increased MAPK pathway activity may predict for sensitivity to MEK inhibitor therapy. Whilst *BRAF* and *RAS* mutations vary across cell lines that are sensitive to MEK inhibition, a MEK 18 gene functional activation signature score was previously found to be consistently elevated in selumetinib sensitive cell lines, and was higher in *BRAF* mutant vs wild-type melanomas<sup>19</sup>. These 18 genes are *DUSP4*, *DUSP6*, *ETV4*, *ETV5*, *PHLDA1*, *SPRY2*, *ELF1*, *FXRD5*, *KANK1*, *LGALS3*, *LZTS1*, *MAP2K3*, *PROS1*, *S100A6*, *SERPINB1*, *SLCO4A1*, *TRIB2*, and *ZFP106*. After refining the score to 6 genes (*DUSP4*, *DUSP6*, *ETV4*, *ETV5*, *PHLDA1*, *SPRY2*), based on reproducibility across tumour types, the MEK 6 gene score has been shown to be higher in *KRAS* mutant than *KRAS* wild-type NSCLC<sup>20</sup>. This suggests that known

activating mutations in the MAPK pathway are associated with higher MEK gene signature scores. Indeed, all the components of the 6 gene score are known transcriptional targets of the MEK-ERK pathway <sup>21,31-36</sup>. Furthermore 4 of 6 of these genes (*DUSP4*, *DUSP6*, *PHLDA1* and *SPRY2*) are negative regulators of ERK pathway activity, forming part of a regulatory feedback loop <sup>21,31,33,36,37</sup>. Consistent with the ability of mutant *NRAS* to activate MEK-ERK signalling, our results suggest that *NRAS* mutant melanoma is associated with a higher MEK 6 gene expression score than *NRAS* wild-type melanoma (Figure 1B). It is interesting to note that the second highest MEK 6 gene score was in a melanoma found to have two concurrent *NRAS* mutations, Q61K and Q61R (Supplementary table 4, patient DM077). However, neither *NRAS* mutation status or VAF analysis was sufficiently discriminating to judge dependence on the MAPK pathway and thus potential sensitivity to MEK inhibition. We then assessed the MEK score with respect to patient outcome, and here the data suggested that a higher MEK score did predict for sensitivity to selumetinib plus docetaxel combination therapy, but not docetaxel therapy alone (Figure 2). To strengthen this conclusion, it would have been preferable to obtain on-treatment biopsies, to confirm that MEK-ERK was indeed inhibited by selumetinib. We also acknowledge that there were only minor differences in MEK signature score by *NRAS* mutation and clinical response status, limiting the utility of this score as a biomarker for MEK inhibitor response in melanoma. Further data would be required to confirm this trend and fully characterise an optimal threshold.

We report here that two components of the MEK 6 gene score, *ETV4* and *DUSP4*, were expressed at significantly higher levels in melanomas of responders to

docetaxel plus selumetinib compared with those who progressed at first assessment. This difference was not found in the docetaxel plus placebo arm. This suggests the possibility that DUSP4 and ETV4 may be potential biomarkers of sensitivity to MEK inhibition. We wished to extend this observation by ascertaining whether DUSP4 or ETV4 might also influence the response of wild-type *BRAF* melanoma cells to MEK inhibition. ETV4 is a member of the polyomavirus enhancer activator 3 (PEA3) sub-family of the Ets transcription factor family and regulates genes that promote metastasis<sup>38</sup>. As well as inducing ETV4 expression, ERK1/2 promotes ETV4 activation by phosphorylation and sumoylation<sup>34,39,40</sup>. Previous studies reported inconsistent findings regarding the contribution of ETV4 to cell survival<sup>41,42</sup>. Our data indicate that ETV4 depletion inhibited cell survival of *BRAF* wild-type melanoma cells, but did not influence response to MEK inhibition.

DUSP4 was the only other component of the MEK 6 gene score that was expressed at significantly higher levels in the melanomas of patients who responded to selumetinib and docetaxel, compared with those who progressed at first assessment (Table 1). DUSP4 dephosphorylates and thus inactivates ERK1/2 in the nucleus, and may also act on the JNK and p38 pathways<sup>43</sup>. There is conflicting evidence regarding the significance of DUSP4 expression in cancer. DUSP4 upregulation has been reported in *KRAS* mutant rectal cancer<sup>44</sup> and higher DUSP4 levels have been found in melanoma cell lines compared with normal human epidermal melanocytes<sup>45</sup>. Conversely, DUSP4 levels are higher in indolent ovarian serous borderline tumours compared with more aggressive serous carcinomas<sup>46</sup>, and silencing of DUSP4 plays a key role in the development of glioblastomas<sup>47</sup>, suggesting a tumour suppressor role. *In vitro*, DUSP4 knockdown increases growth of EGFR mutant lung

adenocarcinoma cell lines, whereas in colorectal cancer cell lines DUSP4 overexpression results in increased proliferation<sup>48</sup>. There is also conflict in the literature regarding the significance of DUSP4 for predicting response to anti-cancer therapy. Higher DUSP4 expression has been found to correlate with resistance to anti-EGFR antibody cetuximab in patients with metastatic colorectal cancer, although this may simply reflect the presence of *KRAS* mutations that activate RAS-MAPK<sup>49</sup>. Conversely, lower DUSP4 expression in breast cancer was reported to be associated with reduced response to neoadjuvant chemotherapy, and in breast cancer cell lines, DUSP4 depletion increased resistance to docetaxel and other cytotoxic drugs, while overexpression increased chemotherapy-induced apoptosis<sup>50</sup>.

Our data are consistent with findings that endogenous DUSP4 levels vary between melanoma cell lines *in vitro*<sup>51</sup>. Relative over-expression of DUSP4 and ETV4 in SK-MEL-23 compared with CHL-1 may reflect the fact that SK-MEL-23 cells harbour amplified wild-type *BRAF*<sup>52</sup>. Both cell lines used in this study harbour wild-type *NRAS*; given that ~60% of patients in the clinical DOC-MEK study had *NRAS* mutant melanoma, it may be informative to assess the contribution of DUSP4 and ETV4 to MEK inhibitor response in *NRAS* mutant cell lines. In the Broad-Novartis Cancer Cell Line Encyclopedia (CCLE) database melanoma cell lines express the highest level of DUSP4 mRNA of all cancer cell lines tested, likely reflecting the importance of RAS-RAF-MEK activation in melanomas ([www.broadinstitute.org/ccle](http://www.broadinstitute.org/ccle)). Of relevance to our study, CCLE data indicate that high DUSP4 expression is predictive of increased sensitivity to selumetinib, with an odds ratio of 2.3<sup>53</sup>. Similarly, in a panel of pan-negative (wild-type *BRAF*, *NRAS*, *KIT*, *GNAQ* and *GNA11*) melanoma cell lines, cells expressing higher levels of DUSP4 have been reported to show significantly

greater sensitivity to MEK inhibition (Hutchinson et al., 2015). This correlates with our finding that CHL-1 cells that express lower DUSP4 were less sensitive to selumetinib than the higher expressing SK-MEL-23 cell line (Figure 4E). We further found that DUSP4 expression was reduced by MEK inhibition, confirming that DUSP4 levels are regulated by the MAPK pathway <sup>21</sup>. Consistent with this, and given that DUSP4 is a negative regulator of the MAPK pathway, depleting DUSP4 increased survival of CHL-1 cells. DUSP4 depletion did not influence survival of SK-MEL-23 cells, possibly because residual DUSP4 may have been sufficient to maintain MAPK pathway regulation, consistent with data in Figure 4B.

Finally, we showed that siRNA-mediated DUSP4 depletion leads to desensitisation to MEK 1/2 inhibition. This response parallels the finding in the clinical trial, where higher DUSP4 expression associated significantly with response to MEK inhibition. We observed the MEK sensitisation effect in CHL-1 cells, where DUSP4 depletion had influenced cell survival, and also in SK-MEL-23 cells, where DUSP4-depleted cells showed no significant difference in cell survival compared with controls. These results suggest that the effect of DUSP4 on MEK inhibitor sensitivity is likely independent of the effect on cell survival. Thus, while previous studies showed a correlation between DUSP4 expression and MEK inhibitor sensitivity in pan-tumour cell line panels <sup>53</sup>, we report for the first time that this association exists in clinical melanomas, and depleting DUSP4 expression induces resistance to MEK inhibition. These results suggest that DUSP4 is capable of influencing response to drugs that target MEK. Furthermore, given that BRAF amplification reportedly mediates MEK inhibitor resistance <sup>54</sup>, the ability of DUSP4 depletion to sensitise SK-MEL-23 cells suggests that this approach may have merit in the BRAF-amplified population.

However, we recognise the need to be cautious in interpretation of our data, given the small size of the clinical study and *in vitro* analysis. It may be informative to assess the predictive significance of DUSP4 in a larger clinical dataset.

In summary, our findings suggest that DUSP4 plays a direct role in determining cellular response to MEK inhibition. DUSP4 may therefore be not only a biomarker for, but also a potential determinant of, the response of wild-type *BRAF* melanomas to MEK inhibition.

## **Ethical approval and consent to participate**

All patients who participated in the DOC-MEK study provided written informed consent, including consent to use of archival tissue samples and clinical data for exploratory research analysis. The protocol was approved by the NHS Oxfordshire A Research Ethics Committee. The study was conducted according to UK Clinical Trials Regulations and the ICH guidelines of Good Clinical Practice.

## **Consent to publish**

Not applicable.

## **Data availability**

The dataset used and analysed for this manuscript is available from the corresponding author on request.

## **Conflicts of Interest**

AG has consulted for and received honoraria from BMS and Novartis. RB, DRH, AS and PDS are employees of AstraZeneca. RB has shares in AstraZeneca. AS has received honoraria from Gilead, Roche, Janssen and Abbvie, and an unrestricted educational grant from Gilead and Janssen. MRM has consulted for and received research funding from AstraZeneca, GlaxoSmithKline and Roche. VMM has consulted for Boehringer Ingelheim.

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#### **Authors' contributions**

DRH and MRM designed the clinical study. AG, MRM and VMM designed the translational research study. AG, CT, FW, RB, AC, AS, RA, KM, LC, AW, MRM and VMM were responsible for the acquisition of data, including clinical trial management, sample analysis and data interpretation, and laboratory experimental work. AG, FW, RB, DRH, AS, PDS, MRM and VMM were responsible for data analysis. AG, FW, SL and VMM were responsible for statistical analysis. AG, FW and VMM wrote the manuscript, with input and final approval from all authors.

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Supplementary information is available at the British Journal of Cancer's website.



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## Legends to Tables

**Table 1: Expression of individual genes in the MEK 6 gene score in melanomas of patients treated with docetaxel plus selumetinib.** Grey shaded rows: patients with PR/CR to docetaxel plus selumetinib combination treatment; unshaded rows: patients with PD at first assessment. \*Cases where ETV4 expression was below the set limit of detection (mean minus 2 standard deviations), so accuracy is unclear. Mean gene expression in both groups was compared using Student's t-test with two-tailed distribution.

**Table 2: Effect of DUSP4 depletion on response to MEK inhibition in BRAF wild-type melanoma cells.** Table shows mean  $\pm$  SEM fold increase in SF50 values in DUSP4-depleted CHL-1 and SK-MEL-23 cells (n=6 in each case; data analysed using 2-tailed t-test).

## Legends to figures

### **Figure 1: Correlation of *NRAS* mutations with MEK 6 gene signature score.**

A, distribution of mutations detected in samples from 59 patients using the 46 gene cancer panel; B, MEK 6 gene signature scores of patient samples with mutant or wild-type *NRAS*; C, correlation between MEK 6 gene signature score and variant allele frequency of *NRAS*.

### **Figure 2: Correlation of response to docetaxel plus selumetinib with MEK 6 gene signature score.**

A, best overall response for patients treated with docetaxel + placebo comparing CR and PR with SD and PD; B, best overall response for patients treated with docetaxel + selumetinib as in (A), \* $p=0.026$  using 1-tailed t-test; C, derived benefit for patients treated with docetaxel + placebo comparing benefit with no benefit; D, derived benefit for patients treated with docetaxel + selumetinib as in (C), \* $p=0.038$  using 1-tailed t-test, testing the pre-established hypothesis that a higher MEK 6 gene score correlates with better clinical outcome when treated with a MEK inhibitor.

### **Figure 3: MEK inhibition decreases ERK phosphorylation and DUSP4 expression in *BRAF* wild-type melanoma cell lines.**

A, endogenous expression of DUSP4 and ETV4 protein in duplicate whole cell extracts of CHL-1 and SK-MEL-23 cells analysed by Western blotting. Similar results were obtained in  $n=3$  independently-prepared lysates. Faint ~40 kDa band (arrowhead) just below DUSP4 band was not reduced by DUSP4 depletion (see Figure 4B-C) so may be non-specific. B, *DUSP4* and *ETV4* mRNA quantified by qRT-PCR ( $n=3$  independently-

prepared cDNAs). C, CHL-1 and SK-MEL-23 cells were treated with 100 nM selumetinib or 10 nM trametinib for 1 hr before analysis by Western blot. Supplementary Figure 1A-C shows concentration and time-dependence of response to MEK inhibition.

**Figure 4: Depletion of DUSP4 decreases sensitivity to MEK inhibition in *BRAF* wild-type melanoma cell lines.** A and B, quantification of DUSP4 depletion following knockdown by siDUSP4\_1 and siDUSP4\_2 in: left, CHL-1 and right, SK-MEL-23 cells analysed by qRT-PCR (A) and Western blot (B). Arrowhead: probable non-specific band not reduced by DUSP4 depletion. C, duration of DUSP4 knockdown in CHL-1 cells, analysed by Western blotting at 3, 5 and 7 days after transfection. Representative results are shown for Allstars (AS) control siRNA and siDUSP4\_1. ERK is shown as a loading control. D, the effect of DUSP4 depletion by siDUSP4\_1 and siDUSP4\_2 on colony count in: left, CHL-1 and right, SK-MEL-23 cells (mean  $\pm$  SEM of 5 independent experiments). E, the effect of DUSP4 depletion by siDUSP4\_1 and siDUSP4\_2 on sensitivity of: left, CHL-1 and right, SK-MEL-23 cells to MEK inhibitors selumetinib and trametinib (mean  $\pm$  SEM of triplicate values for a single representative experiment). Table 2 shows summary of fold changes in MEK inhibitor sensitivity (pooled data from 6 experiments in each case).

**Table 1: Expression of individual genes in the MEK 6 gene score in melanomas of patients treated with docetaxel plus selumetinib.**

Trial no	Normalised values					
	DUSP4	DUSP6	ETV4	ETV5	PHLDA1	SPRY2
<b>PR/CR</b>						
<b>DM015</b>	10.247701	9.609001	6.947348	10.261135	10.842443	9.402991
<b>DM018</b>	9.992847	11.918447	9.105265	10.150524	12.467503	11.006485
<b>DM038</b>	10.206098	9.126801	8.848189	11.202584	12.294669	10.120284
<b>DM040</b>	10.995507	9.970414	8.865340	10.509355	11.409115	9.252343
<b>DM053</b>	10.402294	9.383720	8.223903	11.722223	11.612109	9.329706
<b>DM055</b>	8.559473	9.822125	8.616266	10.050092	10.655798	10.154635
<b>DM058</b>	10.937206	10.281701	7.224400	10.201668	10.476144	9.656104
<b>DM077</b>	11.535519	11.346413	9.737887	10.313185	11.161509	10.787450
<b>Mean</b>	<b>10.359581</b>	<b>10.182328</b>	<b>8.446075</b>	<b>10.551346</b>	<b>11.364911</b>	<b>9.963750</b>
<b>PD</b>						
<b>DM037</b>	9.043606	10.435792	6.683144*	11.486187	11.667844	9.960695
<b>DM042</b>	8.558301	9.713039	6.402249*	9.079551	10.428238	8.959072
<b>Mean</b>	<b>8.800954</b>	<b>10.074415</b>	<b>6.542696</b>	<b>10.282869</b>	<b>11.048041</b>	<b>9.459883</b>
<b>2 tailed p value</b>	0.046997	0.886749	0.026507	0.689342	0.608309	0.372732



**Table 2: Effect of DUSP4 depletion on response to MEK inhibition in BRAF wild-type melanoma cells.**

<b>Cell line</b>	<b>CHL-1</b>		<b>SK-MEL-23</b>	
<b>MEK inhibitor</b>	<b>Selumetinib</b>	<b>Trametinib</b>	<b>Selumetinib</b>	<b>Trametinib</b>
<b>Fold increase in SF50</b>	6.71 ± 1.30	6.85 ± 2.42	2.94 ± 0.40	2.48 ± 0.44
<b>p-value</b>	0.0072	0.0605	0.0048	0.02

## Dual specificity protein phosphatase DUSP4 regulates response to MEK inhibition in *BRAF* wild-type melanoma

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### Supplementary Data

SupplementaryTables

Genes validated for diagnostic use	Genes presented on a research basis			
BRAF	ABL1	ERBB2	HRAS	NPM1
EGFR	AKT1	ERBB4	IDH1	PTPN11
KIT	ALK	FBXW7	JAK2	RB1
KRAS	APC	FGFR1	JAK3	RET
NRAS	ATM	FGFR2	KDR	SMAD4
PDGFRA	CDH1	FGFR3	MET	SMARCB1
PIK3CA	CDKN2A	FLT3	MLH1	SMO
PTEN	CSF1R	GNAS	MPL	SRC
TP53	CTNNB1	HNF1A	NOTCH1	STK11
				VHL

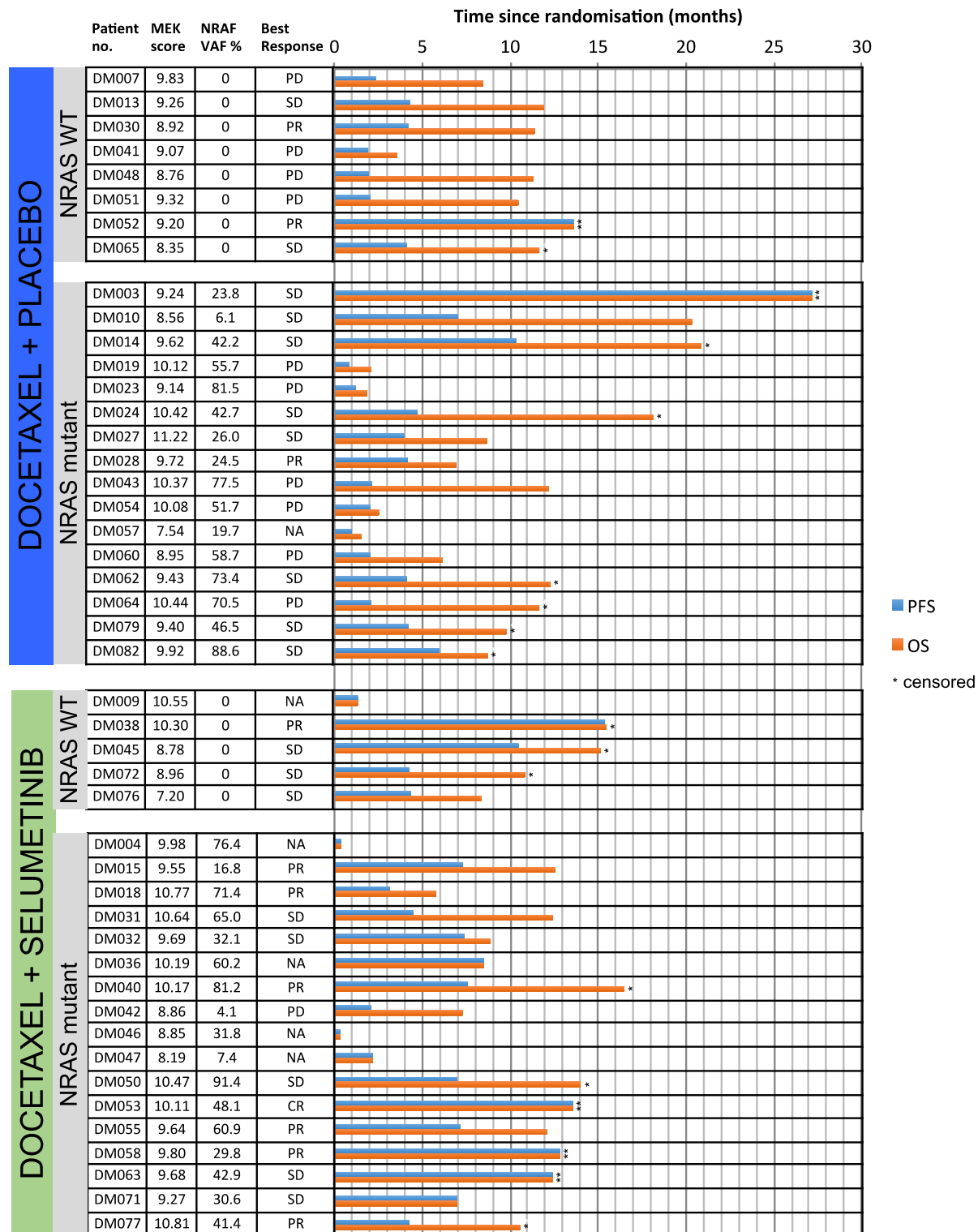
**Supplementary Table 1: Genes in 46 cancer gene panel.** Hotspot mutation detection was performed using IonTorrent Personal Genome Machine. Assays for mutations in nine of these genes have been validated for clinical use and the remaining assays are available for research use.

		No. of mutations detected				
		0	1	2	3	4
DOC-MEK study patient no.	DM001	NRAS				
	DM002	NRAS	ATM	JAK3		
	DM003	NRAS				
	DM004	NRAS	TP53			
	DM006	KIT				
	DM007	FBXW7				
	DM009	HRAS				
	DM010	NRAS				
	DM011					
	DM012					
	DM013					
	DM014	NRAS	TP53	STK11		
	DM015	NRAS				
	DM017					
	DM018	NRAS				
	DM019	NRAS	ATM	CDKN2A	STK11	
	DM023	NRAS	KDR			
	DM024	NRAS	IDH1			
	DM027	NRAS				
	DM028	NRAS				
	DM030	KIT	CTNNB1			
	DM031	NRAS				
	DM032	NRAS	APC	CDKN2A		
	DM036	NRAS				
	DM038					
	DM040	NRAS	TP53			
	DM041					
	DM042	NRAS	ATM			
	DM043	NRAS				
	DM045					
	DM046	NRAS	TP53			
	DM047	NRAS	IDH1			
	DM048	APC				
	DM050	NRAS				
	DM051					
	DM052	APC	KIT	CTNNB1	MET	
	DM053	NRAS				
	DM054	NRAS	IDH1	PIK3CA		
	DM055	NRAS				
	DM056	HRAS				
	DM057	NRAS	APC			
	DM058	NRAS				
	DM060	NRAS	TP53			
	DM061	NRAS				
	DM062	NRAS	TP53			
	DM063	NRAS				
	DM064	NRAS	PTEN			
	DM065	TP53				
	DM067	BRAF(V600E)				
	DM068					
	DM071	NRAS	BRAF(G466R)	CSF1R		
	DM072	ATM	BRAF(K601E)			
	DM073	JAK3	KRAS			
	DM075	NRAS				
	DM076	KDR				
	DM077	NRAS	BRAF(N581S)			
	DM078					
	DM079	NRAS	PIK3CA			
	DM082	NRAS	KDR	FBXW7		

**Supplementary Table 2: Number and type of mutations detected per case:**  
Distribution of mutations detected in samples from 59 patients tested using the 46 gene cancer panel.

No. of mutations	0	1	≥ 2
No. of cases	9	24	24
Median PFS (months, PP population)	4.27	4.39	4.14
Median OS (months, PP population)	10.43	11.65	9.30

**Supplementary Table 3: Summary of mutations detected per sample and patient outcome.** PFS, progression free survival; OS, overall survival; PP, per protocol. There were no significant differences in PFS or OS between patients whose melanomas contained 0, 1 or ≥2 mutations ( $p>0.05$  by one-way ANOVA).

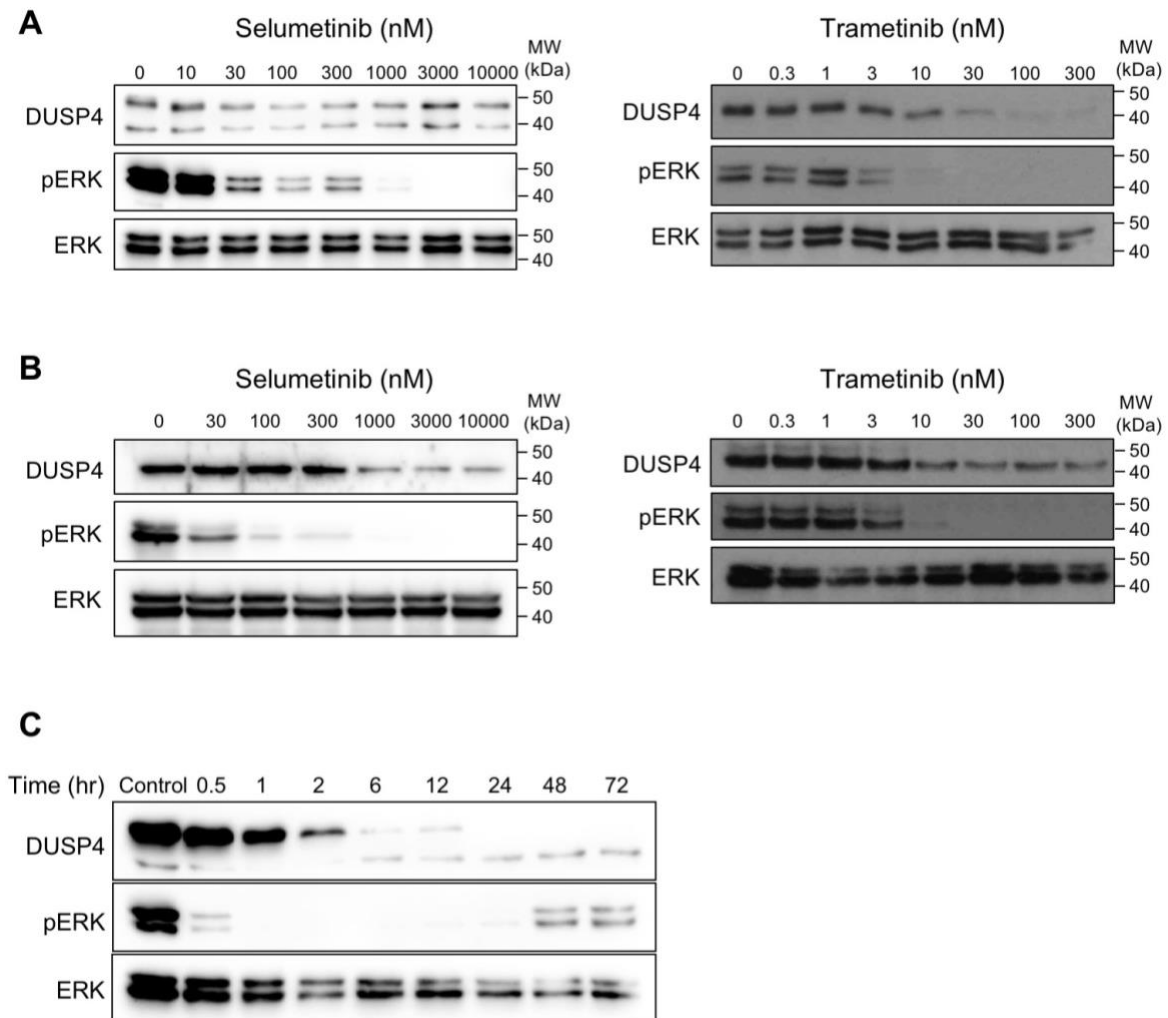


**Supplementary Table 4: Summary of gene expression data correlated with clinical outcome.** *NRAS* status was determined by NGS using the 46 gene cancer panel. WT, wild-type; PD, progressive disease; SD, stable disease; PR, partial response; CR, complete response; NA, not available; c, censored.

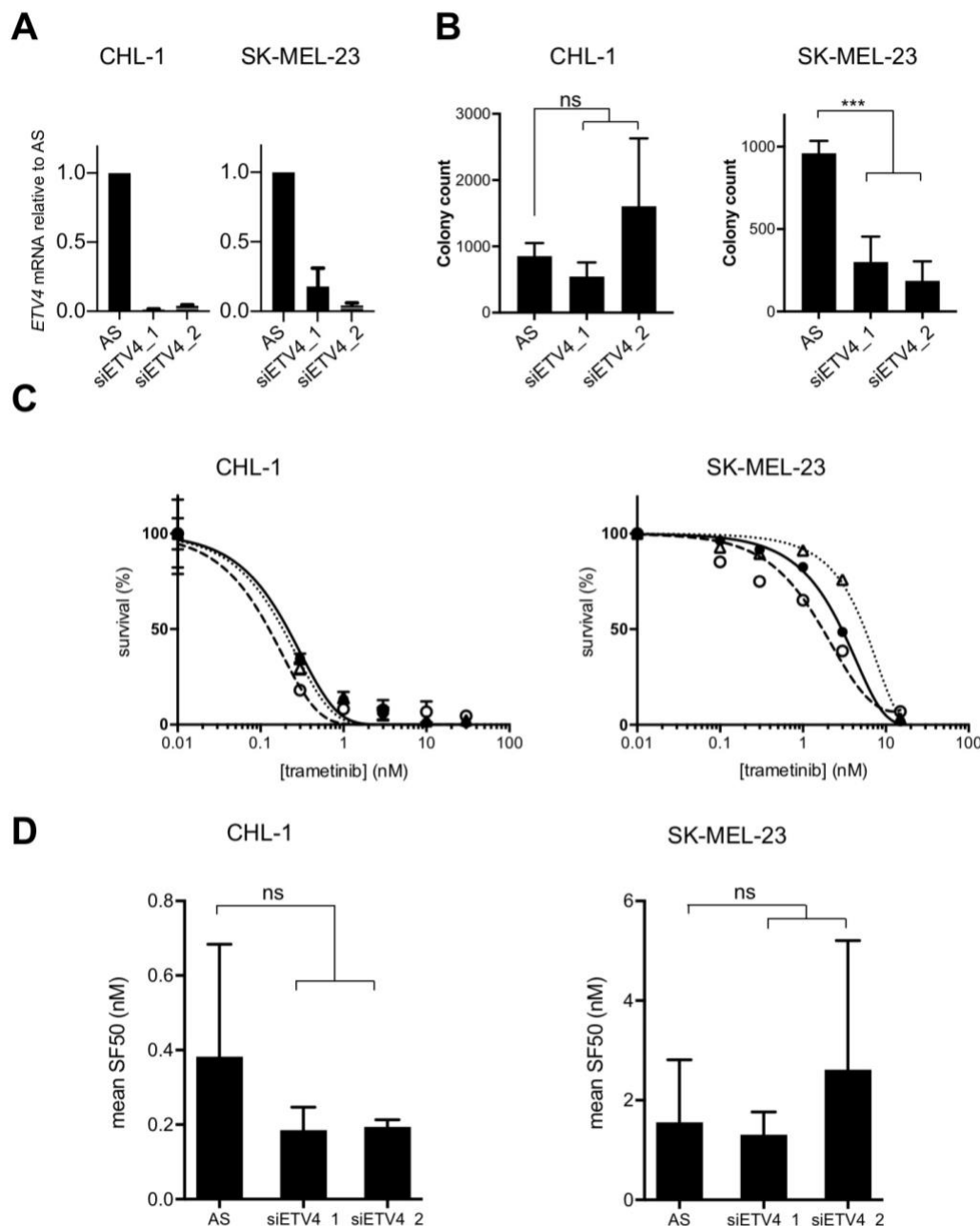
<b>Trial no.</b>	<b>Normalised values</b>					
	<b>DUSP4</b>	<b>DUSP6</b>	<b>ETV4</b>	<b>ETV5</b>	<b>PHLDA1</b>	<b>SPRY2</b>
<b>PR/CR</b>						
<b>DM028</b>	9.887194	10.955445	8.085209	8.990286	10.997901	9.386733
<b>DM030</b>	8.934151	8.182466	8.340894	10.365277	10.358002	7.321323
<b>DM052</b>	10.009136	7.743555	7.967867	11.215295	9.771230	8.513439
<b>Mean</b>	<b>9.610160</b>	<b>8.960489</b>	<b>8.131323</b>	<b>10.190286</b>	<b>10.375711</b>	<b>8.407165</b>
<b>PD</b>						
<b>DM007</b>	9.495310	10.402783	9.047305	9.785372	11.260327	8.960709
<b>DM019</b>	10.720741	10.895120	8.515907	9.836377	10.748692	9.988248
<b>DM028</b>	8.939076	9.317774	7.439848	9.508937	11.026119	8.633325
<b>DM029</b>	8.873473	10.139463	7.626767	10.602202	11.011783	7.857903
<b>DM041</b>	10.544330	7.109423	7.730938	10.536587	10.183001	8.332260
<b>DM043</b>	10.937361	11.041192	9.230746	10.540165	11.073405	9.404785
<b>DM048</b>	6.692641	9.119352	9.004512	9.591143	9.186803	8.991086
<b>DM051</b>	8.481253	9.441539	9.363928	10.993551	10.513146	7.128626
<b>DM054</b>	10.112275	10.847430	6.951539	11.284806	10.981491	10.297288
<b>DM060</b>	10.225808	8.197017	6.634277	9.441036	10.712816	8.513294
<b>DM064</b>	11.035885	10.770720	9.123161	10.884652	11.007795	9.842820
<b>Mean</b>	<b>9.641650</b>	<b>9.752892</b>	<b>8.242630</b>	<b>10.273166</b>	<b>10.700489</b>	<b>8.904577</b>
<b>2 tailed p value</b>	<b>0.969166</b>	<b>0.385887</b>	<b>0.854082</b>	<b>0.869125</b>	<b>0.414625</b>	<b>0.444763</b>

**Supplementary Table 5: Gene expression data for each gene in the MEK 6 gene score in the Docetaxel plus placebo group.** Shaded rows: patients with PR/CR to docetaxel plus placebo; unshaded: patients with PD at first assessment.

## Supplementary Figures



**Supplementary Figure 1. Concentration and time -dependent effect of MEK inhibition on ERK phosphorylation and DUSP4 expression in BRAF wild-type melanoma cells.** A, CHL-1 cells were treated with increasing concentrations of: left, selumetinib (n=1); right, trametinib (n=3, representative result is shown) for 1 hr before analysis by Western blot. B, SK-MEL-23 cells were treated with increasing concentrations of: left, selumetinib (n=1); right, trametinib (n=2, representative result is shown) and analysed as A. C, SK-MEL-23 cells were treated with 10nM trametinib for the indicated times. Control cells were treated with solvent and harvested at 24hr (n=1).



**Supplementary Figure 2: ETV4 depletion does not influence sensitivity to MEK inhibition in BRAF wild-type melanoma cells.** A, Quantification of *ETV4* expression determined by qRT-PCR in: left, CHL-1 and right, SK-MEL-23 cells transfected with control Allstars siRNA (AS) and 2 siRNAs against *ETV4* (siETV4\_1 and siETV4\_2). Data are mean  $\pm$  SEM from triplicate readings from 3 experiments. B, effect of *ETV4* depletion by siETV4\_1 and \_2 on colony count for: left, CHL-1 and right, SKMEL-23 cells. In each case 3000 cells were originally seeded. C, compared with control Allstars siRNA transfectants (closed circles), *ETV4* depletion using siETV4\_1 (dashed line, open circles) and \_2 (dotted line, open triangles) did not influence cell survival in response to trametinib in: left, CHL-1 and right, SK-MEL-23 cells. Values represent mean  $\pm$  SD % survival in triplicate dishes for each condition. D, Summary of effect of *ETV4* depletion on SF50 values for trametinib in: left, CHL-1 and right, SK-MEL-23 cells. Values represent mean  $\pm$  SEM of triplicate values from three independent experiments for both cell lines.