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AUTHOR MANUSCRIPT
Accepted for publication in a peer-reviewed journal

Published as: *Cell*. 2010 May 28; 141(5): 746–747.

Role for IGFBP7 in Senescence Induction by BRAF

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In their recent Matters Arising, Scurr et al. (2010) questioned several of our conclusions regarding the role of IGFBP7 in BRAFV600E-mediated senescence induction. In our original study in *Cell* (Wajapeyee et al., 2008), we used a genomewide RNA interference (RNAi) screen to identify 17 genes required for an activated BRAF oncogene (BRAFV600E) to block proliferation of primary melanocytes and melanoma cells. One of these genes encodes a secreted protein, IGFBP7, which we showed has a central role in BRAFV600E-mediated senescence and apoptosis. Here, we reproduce several of the key findings of our earlier study and present new results that substantiate our original claims.

In our original study (Wajapeyee et al., 2008), we showed that expression of BRAFV600E in primary melanocytes increases synthesis and secretion of IGFBP7, which then acts through an autocrine/paracrine pathway to induce senescence. BRAFV600E-mediated induction of IGFBP7 expression was directly demonstrated in six independent experiments. By contrast, Scurr et al. (2010) claim in their Matters Arising that BRAFV600E results in decreased IGFBP7. We introduced BRAFV600E into cultured human melanocytes by retroviral transduction, and in a series of new experiments now also show that transient transfection of a BRAFV600E-expression plasmid results in increased IGFBP7 (Figure S1A). Furthermore, BRAFV600E-mediated induction of IGFBP7 is, as expected, lost following addition of the MEK inhibitor U0126, which blocks BRAF-MEK-ERK signaling. Transfection of a BRAFV600E-expression plasmid into melanocytes also induces expression of a co-transfected *IGFBP7* reporter plasmid (Figure S1B).

Originally, we showed that BRAFV600E transcriptionally activates other genes involved in senescence or apoptosis including *PEA15, SMARCB1* and *BNIP3L* (Wajapeyee et al., 2008). By contrast, Scurr et al. (2010) claim that following introduction of BRAFV600E into primary melanocytes, PEA15, SMARCB1, BNIP3L and p53 protein levels are reduced. Their p53 result is particularly surprising because activated oncogenes induce a DNA damage response, which is expected to elevate p53 levels. Indeed, in direct contrast to Scurr et al. (2010), another study has shown that BRAFV600E increases p53 levels in melanocytes (Yu et al., 2009). To confirm our original conclusions, we performed a new immunoblot

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Supplemental Data Supplemental Data include Supplemental Experimental Procedures and one figure and Supplemental References and can be found with this article online.

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experiment, which shows that BRAFV600E activates the DNA damage response and markedly upregulates expression of PEA15, SMARCB1, BNIP3L and p53 in primary melanocytes (Figure S1C).

Following the primary screen, we performed 11 independent experiments demonstrating that the BRAFV600E-mediated block to cellular proliferation requires IGFBP7 (Wajapeyee et al., 2008). These experiments involved two different cell types, two unrelated IGFBP7 short hairpin RNAs (shRNAs), and five different assays related to cellular proliferation. Finally, we showed that addition of IGFBP7 to melanocytes is sufficient to induce senescence.

In their Matters Arising, Scurr et al. (2010) performed RNAi experiments and did not find a role for IGFBP7 in BRAFV600E-mediated senescence. To investigate this discrepancy, we repeated several of our original experiments (Wajapeyee et al., 2008), which confirmed that senescence in melanocytes is substantially reduced following shRNA-mediated knockdown of IGFBP7 (Figures S1D,E).

Scurr et al. (2010) suggested that our use of drug selection to introduce BRAFV600E and shRNAs may have inadvertently selected for senescence-resistant cells. To address this concern, we transduced melanocytes with the BRAFV600E-expressing retrovirus in the absence of drug selection. IGFBP7 knockdown was performed using two unrelated small interfering RNAs (siRNAs) in the absence of drug selection, and induction of $p16^{INK4a}$ was analyzed as a marker of senescence. As expected, BRAFV600E results in enhanced expression of $p16^{INK4a}$ as well as IGFBP7 (Figure S1F). Moreover, the two IGFBP7 siRNAs substantially reduced IGFBP7 levels resulting in loss of $p16^{INK4a}$ induction.

Although we do not understand the failure of Scurr et al. (2010) to observe a requirement for IGFBP7 in the induction of senescence by the BRAF oncogene, it is clear that the populations of cells in which senescence is being analyzed are markedly different in the two studies. We analyzed senescence in BRAFV600E-expressing cells that contain elevated levels of IGFBP7, PEA15, SMARCB1, BNIP3L and p53; by contrast, in Scurr et al. (2010) the cells analyzed for senescence contained reduced levels of these factors.

Finally, the inability of Scurr et al. (2010) to observe a requirement for IGFBP7 is essentially a negative result; none of the experiments presented in Figure 3 of their Matters Arising included as a positive control an shRNA that knocks down a gene required for senescence induction. Scurr et al. (2010) presented elsewhere an experiment purporting to show that knockdown of both p53 and pRb abrogates BRAFV600E-mediated senescence (Figure S2). However, examination of their results reveals that the level of BRAFV600E is substantially lower in the p53, pRb double-knockdown cells than in the control cells, which could account for the apparent difference in senescence induction.

In our original study (Wajapeyee et al., 2008), we showed by immunohistochemical analysis that IGFBP7 expression is lost at high frequency in primary melanoma cells containing BRAFV600E. Subsequently, we showed that in metastatic melanomas IGFBP7 is lost at an even higher frequency and is independent of BRAF mutational status (Wajapeyee et al., 2009). Scurr et al. (2010) analyzed IGFBP7 expression in human metastatic, but not primary, melanoma samples and failed to observe a correlation with the status of BRAF, which is in agreement with our metastatic melanoma results. To provide additional support for our original conclusions, we analyzed 14 new primary melanomas and found that IGFBP7 is expressed in all six primary melanomas containing wildtype BRAF but not in any of eight primary melanomas containing BRAFV600E (Figure S1G). As in our previous studies, we also provide independent support for the immunohistochemical results using bisulfite sequencing to determine the methylation status of the *IGFBP7* promoter (Figure S1H).

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Scurr et al. (2010) found that IGFBP7 is expressed in virtually all BRAFV600E-containing benign melanocytic lesions (nevi) in agreement with our original immunohistochemical analysis (Wajapeyee et al., 2008). Using tissue arrays, Scurr et al. (2010) also found that IGFBP7 expression is not detectable in ~50% or more of the metastatic melanoma samples containing BRAFV600E or wildtype BRAF. The Figure 2E legend in the Scurr et al. (2010) Matters Arising states that the median expression value for IGFBP7 is zero for both BRAFV600E-containing and wildtype BRAF-containing metastatic melanoma samples, which is in excellent agreement with our immunohistochemistry results (Wajapeyee et al., 2009). In summary, both our results and those of Scurr et al. (2010) show that IGFBP7 is expressed in primary melanocytes and benign nevi, and that IGFBP7 expression is lost in a high percentage of both BRAFV600E-containing and wildtype BRAF-containing metastatic melanomas.

In our original study in *Cell*, we found that loss of IGFBP7 expression correlates with the presence of the BRAFV600E mutation in human melanoma cell lines (Wajapeyee et al., 2008). Scurr et al. (2010) also observed frequent loss of IGFBP7 expression in human cancer cell lines but without a correlation with BRAF status. However, whereas we analyzed only human melanoma cell lines, Scurr et al. (2010) analyzed a diverse collection of melanoma and non-melanoma cell lines as well as primary cells. For example, of the 10 cell types containing wildtype BRAF and another oncogene RAS analyzed by Scurr et al. (2010), only one (NM138) is a human melanoma cell line. The focus of our original study was melanoma and not other cancers and thus the results of Scurr et al. (2010) using non-melanoma cell lines are not directly relevant to our study or its conclusions. Notably, however, two other laboratories have found, analogous to our results in melanoma, that in primary colorectal cancers there is a loss of IGFBP7 expression that correlates with the presence of an activating BRAF mutation (Hinoue et al., 2009; Suzuki et al., 2009).

In summary, here we provide new results confirming several of the main conclusions of our original study in *Cell* (Wajapeyee et al., 2008), including: (i) the transcriptional induction of IGFBP7 by BRAFV600E, (ii) the requirement of IGFBP7 for BRAFV600E-mediated senescence, and (iii) the frequent loss of IGFBP7 expression in BRAFV600E-containing primary melanomas. Collectively, these results implicate IGFBP7 as a tumor suppressor protein in melanomas.

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