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BCL2A1 is a lineage-specific antiapoptotic melanoma oncogene that confers resistance to BRAF inhibition

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Although targeting oncogenic mutations in the BRAF serine/threonine kinase with small molecule inhibitors can lead to significant clinical responses in melanoma, it fails to eradicate tumors in nearly all patients. Successful therapy will be aided by identification of intrinsic mechanisms that protect tumor cells from death. Here, we used a bioinformatics approach to identify drug-able, “driver” oncogenes restricted to tumor versus normal tissues. Applying this method to 88 short-term melanoma cell cultures, we show that the antiapoptotic gene BCL2A1 is recurrently amplified in ~30% of melanomas and is necessary for melanoma growth. BCL2A1 overexpression also promotes melanomagenesis of BRAF-immortalized melanocytes. We find that high-level expression of BCL2A1 is restricted to melanoma due to direct transcriptional control by the melanoma oncogene MITF. Although BRAF inhibitors lead to cell cycle arrest and modest apoptosis, we find that apoptosis is significantly enhanced by suppression of BCL2A1 in melanomas with BCL2A1 or MITF amplification. Moreover, we find that BCL2A1 expression is associated with poorer clinical responses to BRAF pathway inhibitors in melanoma patients. Cotreatment of melanomas with BRAF inhibitors and obatoclax, an inhibitor of BCL2A1 and other BCL2 family members, overcomes intrinsic resistance to BRAF inhibitors in BCL2A1-amplified cells in vitro and in vivo. These studies identify MITF-BCL2A1 as a lineage-specific oncogenic pathway in melanoma and underscore its role for improved response to BRAF-directed therapy.

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igh-resolution somatic copy number and genome sequencing studies of various cancers have identified key driver mutations that form the basis for rationally targeted therapeutics. In melanoma, the most commonly mutated molecule, the protein kinase BRAF gene, is mutated in ~50% of cases. The majority of BRAF mutations result in the substitution of valine by glutamic acid at position 600 (termed V600E), leading to a ~500-fold increase in its kinase activity (1). BRAF(V600E) promotes oncogenesis through activation of the MEK1/2 kinases and the MAPK signal transduction cascade. BRAF has been shown by overexpression and knockdown experiments to be a critical mediator of melanomagenesis. Introduction of mutated BRAF into immortalized melanocytes leads to anchorage-independent growth and tumors in mice. However, oncogenesis induced by BRAF requires other genetic alterations, because oncogenic BRAF induces cellular senescence in primary melanocytes. In mice, dysregulation of BRAF, in cooperation with inactivation of the tumor suppressors Pten or Ink4a, leads to development of melanoma with short latency (2, 3).

Conversely, suppression of BRAF by RNA interference or small molecule inhibitors leads to cell cycle arrest and apoptosis in preclinical models (4–7). BRAF mutations generally predict response to the BRAF inhibitor vemurafenib (PLX4032), yet some BRAF mutant melanoma cell lines are relatively resistant (8–10). Treatment of most patients whose tumors have the BRAF(V600E)

mutation also leads to tumor regression and improved survival (11). However, the duration of such responses is highly variable and virtually all patients eventually relapse (11–13), indicating that resistance mechanisms limit both the magnitude and duration of clinical response.

Here we undertook an integrated bioinformatic and functional analysis to identify genomically amplified therapeutic targets in melanoma and other malignancies. We identified the antiapoptotic factor BCL2A1 as a unique melanoma oncogene located on chromosome 15q. This region is significantly amplified in ~30–40% of melanomas by large-scale copy number analyses and was previously observed to correlate with resistance of melanomas to chemotherapy (14). Unexpectedly, we find that high-level expression of BCL2A1 is largely restricted to melanomas compared with other tumor types. The lineage-specific expression was attributable to its direct regulation by the melanoma oncogene MITF. BCL2A1 is essential for survival in those melanomas in which it is amplified, and its overexpression is shown to promote tumorigenesis in cooperation with BRAF(V600E). Although BRAF inhibitors lead to cell cycle arrest and modest apoptosis, apoptosis is significantly enhanced by suppression of BCL2A1 in melanomas harboring BCL2A1 or MITF amplification. Finally, the combination of a BRAF inhibitor and obatoclax, an inhibitor of BCL2 family members including BCL2A1 currently in clinical trials, enhances apoptosis and tumor regression in vitro and in vivo.

Results

Bioinformatic Analysis Identifies Targets of Genomic Amplification

High-resolution somatic copy number amplifications combined with gene expression profiles have been previously applied to identify causal oncogenes in a variety of malignancies (15–21). However, considerable obstacles exist to translation of these analyses to the clinic. Reasoning that the ability to identify amplified genes that are restricted to tumor cells compared with host tissues could aid the development of targeted therapy with decreased risk of toxicity, we performed a bioinformatics screen for candidate oncogenes in several tumor types, including breast.
amplified between the two analyses (Fig. L4 and SI Appendix, Fig. S1A), including the lineage-specific transcription factor MITF, consistent with its previously described amplification (21). Of particular interest was BCL2A1, one of six antiapoptotic BCL2 family members that has not been previously described as a human oncogene (24). Other BCL2 family members have been described as oncogenes by amplification or translocation, and several approaches to pharmacologically target these proteins, including BCL2A1, are in clinical development (25–29). To confirm these results, we also evaluated an independent microarray dataset (30). BCL2A1 and MITF mRNA were higher in melanoma compared with skin, which predominantly consists of nonmelanocytes (SI Appendix, Fig. S2A). To confirm that BCL2A1 genomic amplification occurs within primary tumor specimens, in addition to the early melanoma cultures we examined above, we examined data from The Cancer Genome Atlas Project (www.broadinstitute.org/igv/home). This dataset demonstrated that both BCL2A1 and MITF were amplified significantly only in melanoma and that 30.8% of primary melanoma biopsies had BCL2A1 amplification. The genomic region 76–100 Mb of chromosome 15q encompassing BCL2A1 was amplified in 28 of 88 melanoma cell lines (31.8%), early passage tumors, and primary specimens evaluated (GISTIC analysis, q = 6.7 × 10−6) (31, 32) (Fig. 1B). Because SNP arrays are only semiquantitative with respect to copy number, we confirmed BCL2A1 copy number using genomic quantitative PCR (SI Appendix, Fig. S3A). Genomic amplification of BCL2A1 was observed in melanoma and was not seen in other tumor types (SI Appendix, Fig. S1B). Different parts of the amplicon were mainly coamplified, with an 80% overlap among samples amplified at different loci in two independent datasets (SI Appendix, Fig. S1 C and D). After filtering undetectable or weakly expressed genes (SI Appendix, Fig. S1E), only four genes within the 15q amplicon were expressed twofold or greater in amplified versus unamplified cells, including BCL2A1 (Fig. 1B). BCL2A1 mRNA (SI Appendix, Fig. S3B) or protein (SI Appendix, Fig. S3D) correlated well with its amplification level. There was a significant correlation between mRNA expression and protein expression (Pearson correlation R = 0.88, P = 0.03). We evaluated whether BCL2A1 or other candidate genes were required for proliferation of an amplified melanoma cell line, M14. Knockdown of BCL2A1 by siRNA significantly reduced proliferation, whereas knockdown of each of the other genes did not affect growth (Fig. 1C), despite >80% knockdown of mRNA (SI Appendix, Fig. S4A) or protein (SI Appendix, Fig. S4B). Similar results were seen in an additional 15q-amplified cell line (SI Appendix, Fig. S4C). We were unable to reliably detect Cathepsin H (CTSH) by validated antibodies, although mRNA was significantly suppressed at 72 h. Thus, we cannot exclude the possibility that CTSH is functionally required for the growth of 15q-amplified melanoma cells, in addition to BCL2A1, despite the absence of a phenotype under conditions where its mRNA levels are significantly suppressed. We did not detect any relationship between BCL2A1 amplification and MITF amplification (Fisher’s exact test, P = 0.06) or BRAF mutations (Wilcoxon test, P = 0.30), although MITF amplification correlated with higher BCL2A1 expression (see below).

**BCL2A1 Is Dysregulated in Melanoma.** To examine whether BCL2A1 expression was increased in melanomas compared with normal melanocytes (and not simply a marker of the melanocytic lineage), we stained primary human melanomas with a BCL2A1-specific monoclonal antibody (SI Appendix, Fig. S5). In a representative case, we observed that BCL2A1 was highly expressed in the melanoma but had much lower expression in normal melanocytes of adjacent skin (Fig. 2A). Using a tumor progression tissue array consisting of benign nevi, primary cutaneous melanomas, and melanoma metastases, we observed no or low expression in 70% of nevi (n = 27), and no nevi had robust (3+) staining (Fig. 2B and Materials and Methods). In contrast, robust expression was observed in 72% of primary melanomas (n = 60, P < 0.0001 for nevi versus melanoma, Fisher’s exact test). A comparison of an independent set of primary melanoma and benign skin nevi (30) also demonstrated significantly increased expression of BCL2A1 in melanoma (P = 5.4 × 10−17, Wilcoxon rank sum test). We also
examined whether expression of BCL2A1 predicted prognosis. We found that in a series of stage III and stage IV melanomas, high BCL2A1 mRNA (Fig. 2C) or protein (Fig. 2D and SI Appendix, Fig. S6) expression were associated with decreased survival after diagnosis. Collectively, these data suggest an important role of BCL2A1 dysregulation in melanoma pathogenesis.

**BCL2A1 Is a Melanoma Oncogene.** To evaluate the functional requirement for BCL2A1 in melanoma, we suppressed BCL2A1 by siRNA and evaluated growth in a colony formation assay, a breast cancer cell line, MCF7, which does not express BCL2A1, was not sensitive to BCL2A1 knockdown (Fig. 3A). However, melanomas with 15q amplifications were dependent on BCL2A1. In contrast, BCL2A1-nonamplified melanomas were not dependent on BCL2A1, despite efficient knockdown (SI Appendix, Fig. S3C). BCL2A1 knockdown by shRNA (SI Appendix, Fig. S4D) also significantly impaired the tumorigenicity of M14 melanoma cells in mouse xenografts (Fig. 3B). To evaluate whether BCL2A1 can promote transformation, we used a previously described assay using genetically immortalized human melanocytes that exhibit soft-agar clonogenic growth upon oncogenic transformation (21). BCL2A1 expression (Fig. 3C) together with BRAF(V600E) efficiently promoted growth of these cells in soft agar compared with control-infected cells (Fig. 3D).

**Lineage Restricted BCL2A1 Expression in Melanoma Owing to Its Direct Regulation by MITF.** We next evaluated expression of BCL2A1 across a panel of melanoma and other tumor cell lines. The expression of BCL2A1 was strikingly restricted to melanomas compared with tumors from other tissue types (Fig. 4A; $P = 6.05 \times 10^{-10}$, Wilcoxon rank sum test). These results were confirmed in other datasets (Fig. 4B; $P = 2.99 \times 10^{-16}$, t test). Consistent with prior reports, moderate expression was found in some lymphoid malignancies (33) in a larger collection of 319 cancer cell lines, although BCL2A1 was not amplified in these tissues (SI Appendix, Fig. S1B).

We identified transcription factors whose expression was associated with BCL2A1 expression (SI Appendix, Table S2). Levels of MITF were strongly correlated with BCL2A1 expression (Pearson correlation 0.56, $P = 4.4 \times 10^{-9}$). Moreover, BCL2A1 was correlated with other genes known to be directly regulated by MITF, such as SILV, TYR, and DCT (34–36), but not other highly expressed genes (SI Appendix, Fig. S7C). MITF itself has been previously identified as a lineage-specific oncogene that regulates melanoma growth and survival (21). Although it is amplified in only $\sim 15–20\%$ of melanomas, it is essential for the survival of most melanomas. Although MITF directly regulates the BCL2 gene (37), which is functionally related to BCL2A1. MITF deficiency produces a significantly more severe melanocyte defect (embryonic melanocyte lineage loss) than BCL2 deficiency (postnatal melanocyte loss), suggesting that other MITF target genes contribute importantly to MITF’s melanocytic survival phenotype. Unlike other mammalian species, the mouse genome contains four closely related homologs of BCL2A1 (38), precluding straightforward analysis of its genetic contribution to the murine melanocyte lineage.

To evaluate whether MITF is sufficient to activate BCL2A1, we treated primary melanocytes with the cAMP agonist forskolin, which increased MITF expression within 2–3 h (39) (Fig. 4C). BCL2A1 was also induced by forskolin and was delayed relative to MITF, similar to another direct MITF target, TRPM1. Forced lentiviral overexpression of MITF in melanocytes was also sufficient to induce BCL2A1 mRNA (SI Appendix, Fig. S7A). To evaluate whether MITF is required for BCL2A1 expression we suppressed endogenous MITF by siRNA in primary melanocytes and several melanoma cell lines (Fig. 4D). BCL2A1 mRNA was significantly reduced in all cells examined. Similar results were seen at the protein level using two lentiviral-delivered shRNAs targeting MITF (Fig. 4E). MITF knockdown suppressed BCL2 in some but not all melanocytic cells and did not suppress other
antiapoptotic BCL2 family members (BCL2L1, BCL2L2, or MCL1) (SI Appendix, Fig. S7B)).

We compared the promoters of BCL2A1 in mammalian species (Fig. 4F) and identified putative MITF binding sites (E-boxes), located 7 kb upstream of the transcriptional start site and within the 5' untranslated region. These sequences were found in all species examined except Mus musculus, which as stated above has four distinctly encoded BCL2A1 genes (38). To detect in vivo occupancy of MITF at these binding sites, we performed ChIP using primers spanning each region. Strong MITF binding was detected at −7 kb at levels similar to another MITF target, tyrosinase (Fig. 4G). Weak binding was detected at the 5'UTR site. We also evaluated whether BCL2A1 transcription was dependent on the conserved E-boxes and observed that mutation of the E-box at −7 kb suppressed basal activity of the promoter by 44% compared with the wild-type promoter (Fig. 4H). shRNA targeting of MITF reduced BCL2A1 promoter activity by 50% in an E-box-dependent manner. Conversely, overexpressed MITF increased BCL2A1 promoter activity, also in an E-box-dependent fashion.

BCL2A1 Confers Resistance to BRAF Inhibitors. We evaluated genomic amplification of the presurival BCL2 family in melanoma and other cancer types. Both MCL1 and BCL2A1 were genomically amplified in subsets of melanomas (SI Appendix, Fig. S8), whereas MCL1 is known to be amplified in multiple human tumor types (15), BCL2A1 amplification is seen exclusively in melanoma. Because amplification of BCL2 family members may limit the effectiveness of chemotherapy or targeted therapy, we evaluated whether BCL2A1 could mediate relative intrinsic resistance to BRAF inhibitors (refer to SI Appendix, Table S3 for BRAF mutation and BCL2A1 amplification of cell lines used). In a BCL2A1-unamplified BRAF-mutant cell line, overexpression of BCL2A1 inhibited the antiproliferative effect of PLX4720 (SI Appendix, Fig. S9A) or chemotherapy (SI Appendix, Fig. S10E) and protected from apoptosis (Fig. 5A) but not cell cycle arrest (SI Appendix, Fig. 10 A and B). BCL2A1 did not affect the ability of BRAF pathway inhibitors to suppress ERK activity (Fig. 5B). Conversely, in a BCL2A1-amplified cell line that also contains an oncogenic mutation in BRAF, knockdown of BCL2A1 significantly increased sensitivity to PLX4720 (Fig. 5C and D and SI Appendix, Fig. S9B). Similar results were obtained with the structurally unrelated MEK inhibitor, GSK1120212, diminishing the likelihood of off-target effects (Fig. 5A and D and SI Appendix, Fig. S10D). To further minimize the likelihood of off-target effects, we knocked down BCL2A1 using individual, rather than pooled, siRNAs (SI Appendix, Fig. S9F). The sensitization to apoptosis correlated with the degree of BCL2A1 knockdown by each siRNA. The sensitivity to PLX4720 in a cell line carrying wild-type BRAF and no BCL2A1 amplification was not affected by the knockdown of BCL2A1 (Fig. 5E), despite similar knockdown to BCL2A1-amplified M14 cells (SI Appendix, Fig. S9G). PLX4720 only modestly induced apoptosis, consistent with observations that the drug has predominately a cytostatic, noncytocidal effect on many melanoma cell lines in vitro (4–7). Suppression of BCL2A1 significantly enhanced apoptosis in BRAF-mutant BCL2A1- or MITF-expressing cell lines or short-term cultures, but not in melanomas lacking BCL2A1 or a BRAF-mutant colon cancer cell line (Fig. S5E). We also found that suppression of BCL2A1 by shRNA significantly reduced the number of resistant clones after 2 wk of PLX4720 treatment (SI Appendix, Fig. S9C). MITF knockdown also enhanced overall cytotoxicity of BRAF inhibitors (SI Appendix, Fig. S9 D and E).

Consistent with these genetic data, treatment with a pan-BCL2 family inhibitor, obatoclax, synergistically induced apoptosis of a BCL2A1-amplified cell line (Fig. 5G and SI Appendix, Fig. S11A), whereas ABT-737 (an inhibitor of BCL2, BCL2L1, and BCL2L2 but not of MCL1 or BCL2A1) did not. To evaluate this combination in vivo, we treated mouse xenotransplants of A375 cells (which do not express appreciable BCL2A1) or derivatives expressing BCL2A1 (Fig. 5J) with PLX4720, obatoclax, or the combination for 2 wk (Fig. 5H). PLX4720 modestly decreased the size of the control tumors over this period, whereas tumors
overexpressing BCL2A1 slightly grew in size. Obatoclax alone did not significantly affect the growth of tumors, but in combination with PLX4720 it significantly decreased tumor volume of both BCL2A1-overexpressing and control tumors. Two of five animals treated with the combination had no detectable tumors present after 2 wk. Moreover, we did not observe any overt toxicity of the combination of PLX4720 and obatoclax or significant difference in weight of the animals (*SI Appendix, Fig. S11B*). These data indicate that the combination of BRAF inhibitors and obatoclax may be an attractive therapeutic combination for melanomas with high expression of BCL2A1, either by genomic amplification or dysregulation of its upstream regulator, MITF.

To evaluate the clinical relevance of BCL2A1 in conferring resistance to BRAF inhibitors, we evaluated 19 melanoma patients for whom we have biopsies before treatment to either vemurafenib or the combination of GSK1120212 (MEK inhibitor) and GSK2118436 (BRAF inhibitor) (referred to SI Appendix, Table S4, for patient details). Tumors from patients with objective RECIST responses exhibited significantly lower levels of BCL2A1 expression (*P = 0.03*) compared with those that had no objective response (Fig. 5I). Collectively these data suggest that identification of patients with dysregulated BCL2A1 may have poorer clinical outcomes that may be improved by concomitant treatment with BCL2 antagonists such as obatoclax.

**Discussion**

Small molecule suppression of the BRAF/MEK pathway in melanoma produces clinical responses in a majority of melanoma patients (13), but the responses are variable and all patients eventually relapse. Although recent reports have elucidated several mechanisms by which melanomas acquire resistance to BRAF inhibitors (40–42), it is notable that patients treated with BRAF inhibitors rarely have complete initial responses. We propose that amplification of BCL2A1 or its direct regulator MITF may limit the primary efficacy of BRAF-directed therapy. Although we find a correlation between BCL2A1 expression and sensitivity to BRAF inhibitors in vitro and in patients, larger, prospective trials to evaluate the effect of BCL2A1 dysregulation on BRAF inhibitor efficacy will be necessary. Moreover, it will be necessary to correlate genomic copy number with protein expression in primary melanoma tissues.

Our finding that the well-established melanoma oncogene MITF directly regulates BCL2A1 suggests that it may also contribute to resistance to BRAF inhibitors. MITF is amplified in 15–20% of melanomas, although it is functionally required in a larger group of melanomas, including many that lack MITF amplification (21). Although there are no small molecules that target MITF, targeting downstream pathways such as BCL2A1 may be of clinical utility to this group of melanomas. In contrast, MITF-negative melanomas do not express BCL2A1 and are not sensitive to BCL2A1 suppression.

Previous work has evaluated the role of BCL2 family members in mediating resistance to targeted therapy. In lung cancer, reducing MCL1 expression sensitized epidermal growth factor receptor mutant nonsmall cell lung cancers to MEK inhibitors (43). MEK inhibitors synergized with the BH3 mimetic ABT-737 in BRAF mutant colon cancer cell lines (44). In contrast, we found that ABT-737 did not synergize with PLX4720 in melanoma. These data are consistent with the lack of efficacy of a BCL2 antisense oligonucleotide in patients with melanoma (45). The differences between melanoma and other colon cancer cell lines may be related to the inability of ABT-737 to inhibit BCL2A1 in melanoma. These data indicate that the well-established melanoma oncogene MITF directly regulates BCL2A1 and that it may contribute to resistance to BRAF inhibitors.

**Materials and Methods**

**Cell Lines and Cultures.** Cell lines were from American Type Culture Collection and were maintained as described in SI Appendix, SI Text.
Western Blotting and Immunohistochemistry. Antibodies used were MITF CS hybridoma, BCL2A1 (Cell Signaling Technology), α-tubulin (clone DMA1; Sigma), GAPDH (clone 1F9; Cell Signaling Technology), BLM (Epitomics), ISG20 (Sigma), and HA (3F10; Roche). For immunohistochemistry, tumor arrays were stained with anti-BCL2A1 rabbit monoclonal clone 1639–1–I 50 dilution; Epitomics). Details of tissue microarray, staining, and scoring are described in **SI Appendix, SI Text**.

RNA Isolation, Chromatin Immunoprecipitation, and Quantitative Real-Time PCR. Total RNA was isolated using RNeasy RNA kits (Qiagen) and quantitative real-time PCR was performed on an Applied Biosystems 7700 system. Primers and conditions for PCR are described in **SI Appendix, SI Text**.

**siRNA Delivery and Analysis.** siRNAs SMARTpools (Dharmacon) were transfected into melanomas or primary melanocytes using the lipidoid delivery agent C12-133-B as described in **SI Appendix, SI Text**. Lentivirus was prepared by transfection in 293T cells as described in **SI Appendix, SI Text**.

**Cell Growth and Soft-Agar Assays.** pLEX HA-HA-MYC BCL2A1 (Open Biosystems) was packaged in 293T cells and infected into indicated cells as described in **SI Appendix, SI Text**. Infected pmel1 BRAFV600E cells were plated onto soft agar as described (21) and cell number was estimated with the CellTiter-Glo luminescence assay after 2 wk. All mouse experiments were done in accordance with Institutional Animal Care and Use Committee (IACUC) approved animal protocols at Massachusetts General Hospital.

**Promoter Assays and Luciferase Experiments.** The BCL2A1 promoter was amplified from discarded human foreskin and cloned into the pGL3-Basic vector. Mutagenesis was performed using the QuikChange Mutagenesis Kit (Stratagene). Primer sequences for mutagenesis are indicated in **SI Appendix, SI Text**. Luciferase readings were normalized to cotransfected pRL-CMV Renilla Control.

**Apoptosis and Flow Cytometry Assays.** Cells were reverse-transfected with 25 nM siRNA as above and treated with PLX4720 (Sai Advantum Pharma Limited, GS1120212 (Active Biochem), ABT-737, or obatoclax (Selleck Chemicals) for 48 h. Apoptosis was measured using the Annexin V Apoptosis Kit (Becton Dickenson). Cell cycle analysis was done 12 h after treatment with drug using propidium iodide. Cell cycle phases were estimated using Flow software (Treestar Software).

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