**Abstract**

Melanomas resist conventional chemotherapeutics, in part, through intrinsic disrespect of apoptotic checkpoint activation. In this study, using an unbiased genome-wide RNA interference screen, we identified RhoJ and its effector PAK1, as key modulators of melanoma cell sensitivity to DNA damage. We find that RhoJ activates PAK1 in response to drug-induced DNA damage, which then uncouples ATR from its downstream effectors, ultimately resulting in a blunted DNA damage response (DDR). In addition, ATR suppression leads to the decreased phosphorylation of ATF2 and consequent increased expression of the melanocyte survival gene Sox10 resulting in a higher DDR threshold required to engage melanoma cell death. In the setting of normal melanocyte behavior, this regulatory relationship may facilitate appropriate epidermal melanization in response to UV-induced DNA damage. However, pathologic pathway activation during oncogenic transformation produces a tumor that is intrinsically resistant to chemotherapy and has the propensity to accumulate additional mutations. These findings identify DNA damage agents and pharmacologic inhibitors of RhoJ/PAK1 as novel synergistic agents that can be used to treat melanomas that are resistant to conventional chemotherapies.

**Introduction**

Melanoma, an aggressive and fatal malignancy resistant to current therapies, has increased in incidence and mortality over the last several decades (1). The DNA alkylating agents dacarbazine or temozolomide are still used to treat metastatic melanoma, but only 16% of patients respond with no improvement in overall survival (2). Melanomas are resistant to a spectrum of chemotherapies including cisplatin, paclitaxel, docetaxel, combination chemotherapy, immunotherapy, and even newly developed BRAF inhibitors (3, 4). Melanoma cells acquire the ability to invade adjacent tissues and resist chemotherapy early during their evolution (4), further underscoring the importance of developing more effective treatments for this tumor.

Melanoma cells upregulate multiple pathways that allow them to be intrinsically resistant to apoptosis (4)—this includes the activation of anti-apoptotic factors [inhibitor of apoptosis protein (IAP) family, FLIP], the downregulation of pro-apoptotic genes (APAF-1, BAD, BIM), and the activation of prosurvival pathways (NF-κB, AKT). Despite the fact that only 10% to 20% of melanomas contain p53 mutations, most melanoma tumors contain additional mechanisms to suppress the function of p53, a central regulator of chemoresponsiveness (5). Transcriptional (6) and enzymatic regulators of melanogenesis (7) also modulate melanoma chemoresponsiveness. While extensive studies have identified multiple pathways that control melanoma chemoresistance, this information has not yet led to the development of more effective regimens to treat melanoma.

Synthetic lethal functional genomics screening is an emerging strategy to identify drug targets for the rational design of synergistic agents with selective toxicity toward cancer cells (8, 9). A gene and a drug have a synthetic lethal relationship if mutation or depletion of that gene sensitizes cells to sublethal concentrations of a drug (8, 9). Synthetic lethal screening has been used to identify genes that regulate lung cancer, cervical cancer, and breast cancer chemoresistance (9–11). In this study, we use a systems-level screening approach to identify regulators of melanoma chemoresistance with the goal of discovering pathways that could be the molecular targets of new synergistic chemotherapy regimens. This screen identified RhoJ, a CDC42 homologue that regulates endothelial cell migration and angiogenesis (12), as a novel regulator of melanoma chemoresponsiveness. We find that RhoJ activates PAK1 in response to DNA damage, which then suppresses the ability of ATR to activate its downstream effectors Chk1 and ATF2. ATR suppression ultimately results in decreased DNA damage–induced apoptosis and the increased expression of prosurvival genes. Taken together, these studies uncover a new signaling pathway that coordinately regulates survival and chemoresistance.
Materials and Methods

Cell culture and reagents
MNT-1 cells were a gift of M. Marks (University of Pennsylvania, Philadelphia, PA) and were cultured as described (13). C8161 melanoma cells were obtained from Frank Meyenkens (University of California at Irvine, Irvine, CA). SK-Mel-28 melanoma cells were obtained from the American Type Culture Collection. SK-Mel-28 and C8161 melanoma cells were cultured in RPMI medium with 10% FBS. Darkly pigmented normal human melanocytes were purchased from Cascade Biologics and cultured as recommended by the manufacturer. The genome-wide siRNA library used in these studies and the transfection protocols were previously described (13). Dacarbazine and cisplatin were purchased from Sigma and IPA-3 was purchased from Toecris Biosciences. Cell line verification was conducted by Powerplex genotyping before use.

Antibodies
The antibodies for cleaved PARP, β-actin, p-Ser345-Chk1, Chk1, p-Ser20-p33, p73, phospho-Ser34-NS1, NBS1, tubulin, ATF2, PLK1, PAK1, and phospho-PAK1 (Ser199/204)/PAK2 (Ser192/197) were purchased from Cell Signaling Technology. Rhof (monoclonal from Abnova and polyclonal from Sigma), p53 (rabbit polyclonal, Santa Cruz Biotechnology), Sox10 (goat polyclonal, Santa Cruz Inc.), phospho-Ser490/498-ATF2 (Thermo Scientific), and p-Thr68-Chk2 and claspin (Abcam) were purchased as indicated.

High-throughput transfection protocol
High-throughput transfection was conducted as described (13). MNT-1 melanoma cells in plates were incubated for 72 hours at 37°C/5% CO2, and light-activated dacarbazine (14) was added to make a final concentration of 0.1 mg/mL. After an additional 48 hours of drug incubation, a CellTiter-Glo Reagent (CTG; Promega) was delivered to each well, and the luminescence values for each well were determined. All SMART-pooled siRNAs used in this study were purchased from Dharmacon Inc.

Clonogenicity assays
SK-Mel-28 melanoma cells were transfected with 50 nmol/L pooled siRNAs. Forty-eight hours after transfection, cells were incubated in the presence and absence of 10 μmol/L cisplatin. Seventy-two hours after cisplatin treatment, treated cells were washed with PBS and incubated in fresh media for an additional 5 days to allow colony regrowth. Relative cell numbers were quantified using a sulforhodamine B assay (Sigma).

Flow cytometry
For the cell-cycle analysis, melanoma cells were trypsinized and washed with PBS and then were fixed in 70% ethanol. Cells were washed with PBS after fixation and were stained in a PBS solution containing 40 μg/mL propidium iodide (PI), 0.1 mg/mL RNaseA, 0.1% Triton-X 100. For the apoptosis assays, cells were trypsinized, washed with PBS, and stained with Alexa Fluor 488-Annexin V/PI according to the manufacturer’s protocol (V13245; Invitrogen). After 15-minute staining, cells were subjected to flow cytometric analysis (BD FACScalibur), and the resulting data were analyzed using FlowJo. We used cells that were not treated with cisplatin to establish gating parameters. Cells in the early-stage apoptosis were defined as the PI-negative Annexin V–positive population, whereas late-stage apoptotic cells were defined as the PI-positive Annexin V–positive population.

IPA-3 cisplatin sensitization studies
SK-Mel-28 melanoma cells or melanocytes were plated on 96-well plates. Twenty-four hours after plating, cisplatin and IPA-3 were added as indicated. Forty-eight hours after drug treatment, a CellTiter-Glo kit (Promega) was used to quantify the number of surviving cells. The relative percentage of surviving cells in IPA-3–treated and vehicle-treated samples was measured to calculate the synergy between IPA-3 and cisplatin.

Results

Genome-wide siRNA screen to identify candidate genes that modulate melanoma chemoresponsiveness
We used a previously described (9) Dharmacon siRNA library of 84,508 siRNAs corresponding to 4 unique siRNA duplexes, targeting each of the 21,127 unique human genes arrayed in a one-gene/one-well format (4 siRNAs toward each gene) on 96-well microtiter plates to identify genes that selectively support melanoma cell survival in the presence of dacarbazine (DTIC). MNT-1 cells, a cell line with intermediate resistance to a previously used in vitro genotoxic stress (light-activated dacarbazine; ref. 14), were transfected with 35 nmol/L target siRNA using published protocols (13) and incubated in the presence and absence of an empirically determined sublethal dose of dacarbazine (Fig. 1A). An ATP-dependent luminescence cell viability assay (CellTiter-Glo) was used to quantify the impact of each individual siRNA or siRNA + dacarbazine on cell viability. To identify genes that selectively support cell survival in the presence of dacarbazine, we calculated normalized luminescence values from dacarbazine-treated and -untreated wells for each siRNA and used these values to generate cell viability ratios (Supplementary Table S1). siRNAs with low viability ratios (lower cell numbers in siRNA-transfected, dacarbazine-treated wells than in siRNA-transfected, carrier-treated wells) correspond to dacarbazine synthetic lethal genes. In addition to identifying 140 candidate genes that modulate melanoma chemoresponsiveness (Supplementary Fig. S1A, red dots), we identified an even larger number of siRNAs that appeared to selectively promote cell survival in the presence of dacarbazine (z-score > 4). Closer analysis of these high z-score siRNAs revealed that they all potently induced cell death in the absence of dacarbazine (Supplementary Fig. S1B), whereas the addition of dacarbazine appeared to promote the survival of a fraction of the siRNA-transfected cells. Interestingly, this set of genes was enriched in kinases including BRAF (Supplementary Fig. S1C), the target of new melanoma therapies (15). While BRAF siRNAs potently inhibited ATP accumulation in MNT-1 cells (Supplementary Fig. S1B, red bar), the addition of either dacarbazine or cisplatin slightly inhibited the impact of BRAF depletion on ATP accumulation (Supplementary Fig. S1D). Recent clinical
Figure 1. Genome-wide siRNA screen identifies core regulators of melanoma chemoresistance. A, identification of regulators of dacarbazine resistance in MNT-1 cells. MNT-1 melanoma cells were transfected with a genome-wide siRNA library. Seventy-two hours posttransfection, duplicate plates were incubated in the presence and absence of a sublethal dose of dacarbazine for an additional 48 hours. CellTiter-Glo values were determined for each well and normalized to internal reference samples on each plate, followed by normalization to the experimental mean for each well calculated from the full dataset. Similarly adjusted luminescence values from drug-treated samples were generated and used to calculate a normalized ratio (drug-treated/untreated). B, identification of gene targets that potently sensitize melanoma cells to cisplatin. SK-Mel-28 (SKM28) melanoma cells were transfected with the indicated siRNA and incubated with cisplatin as indicated. Relative cell number was determined using a CellTiter-Glo assay. The fraction of surviving cells is indicated to highlight the siRNAs that significantly sensitize cells to cisplatin. **P < 0.01 compared with siControl, determined by the Student t test. C, Rho GTPases sensitize melanoma cells to chemotherapy-induced apoptosis. SKM28 and MNT-1 melanoma cells were transfected with 50 nmol/L target siRNAs and incubated in the presence and absence of the 30 μmol/L cisplatin. Twenty-four hours after drug treatment, lysates were prepared and subjected to immunoblotting with cleaved PARP and actin-loading control antibodies. Representative blot is shown. D, clonogenicity assays identify genes that regulate cell survival in the presence of cisplatin. SKM28 melanoma cells were transfected with the indicated siRNAs and incubated with 10 μmol/L cisplatin for 72 hours. Wells were then washed and media were repleted. Relative cell number in cisplatin-treated and untreated samples was quantified using a sulforhodamine B assay and was used to calculate the ratio of surviving cells. **P < 0.01 comparing to siControl, determined by the Student t test. E, RhoJ
trials have shown that the combination of dacarbazine and BRAF inhibitors offers no improvement in survival when compared with patients treated with dacarbazine alone (16), suggesting that DNA damage agents and kinase inhibitors are not necessarily synergistic in the clinical setting. These findings reinforce the importance of establishing a mechanistic rationale for combination chemotherapies before the initiation of clinical trials.

Identification of synthetic lethal genes that sensitize melanoma cells to chemotherapy-induced apoptosis

Dacarbazine induces the methylation of guanine bases, resulting in base mispairings that are ultimately repaired by the DNA mismatch repair system (17). A dacarbazine-based synthetic lethal screening approach was predicted to identify candidate genes that control mismatch repair while also identifying other candidate genes that suppress the cellular response to DNA damage. To identify the subset of candidates that suppress the DNA damage response (DDR), we next sought to identify siRNAs that sensitized melanoma cells to both dacarbazine and cisplatin, an agent that induces the formation of more bulky DNA adducts, which can be repaired by several different DNA repair mechanisms (17).

Initial studies eliminated false-positives present in our candidate list (Supplementary Fig. S2A) and used a pool deconvolution approach to confirm that the observed dacarbazine sensitization phenotypes for our top 12 hits were not a result of RNA interference off-target effects using well-established criteria (Supplementary Fig. S2B; refs. 9, 13, 18). The top 12 siRNAs identified in the screen also modulated dacarbazine resistance in another melanoma cell line (SK-Mel-28 melanoma cells; Supplementary Fig. S2C). In addition, we validated that the siRNAs used in the study effectively inhibited the expression of their target genes (Supplementary Fig. S4A).

Dose–response studies revealed that 7 of the 12 identified siRNAs potently sensitized SK-Mel-28 melanoma cells to cisplatin at low doses (RND2, RhoJ, SMARCE1, PPP1R12C, BLM, PMS2, and ARI4A; Fig. 1B). As predicted, 2 regulators of DNA damage resistance identified in the screen were DNA repair genes (PMS2 and BLM) known to repair damage induced by cisplatin (Table 1; refs. 19, 20). Two other genes (SMARCE1 and PPP1R12C) regulate essential processes that are required for DNA replication: chromatin remodeling (21) and mitosis (22), respectively. Three of the 7 validated genes were Ras superfamily GTPases (RhoJ, ArlHa, Rnd2), implicating a novel role for Ras superfamily GTPases in the DDR. Colony formation assays revealed that depletion of RhoJ, RND2, BLM, PPP1R12C, and SMARCE1 had profound effects on the proliferation/survival of cisplatin-treated SK-Mel-28 cells (Fig. 1B). Once we had identified the subset of candidate genes that potently regulate cell proliferation/survival, we next measured the accumulation of apoptosis markers (cleaved PARP) in siRNA-transfected, drug-treated cells to identify which of these siRNAs sensitize melanoma cells to cisplatin-induced apoptosis. While low-dose cisplatin treatment induced baseline levels of PARP cleavage in MNT-1 and SK-Mel-28 cells treated with control siRNAs (Fig. 1C), low-dose cisplatin induced significantly more PARP cleavage in MNT-1 and SK-Mel-28 cells treated with RhoJ, RND2, ARL4A, PMS2, and BLM siRNAs (Fig. 1C). Of interest, one of the genes that sensitized cells to cisplatin in both cell lines was ARL4A, an Arf-like GTPase that is genetically amplified in melanoma (23). The gene that most potently sensitized cells to cisplatin-induced death was RhoJ. Retrospective analysis of previously published microarray datasets revealed that RhoJ is overexpressed in metastatic melanoma (Supplementary Table S2; ref. 24), implicating a role for RhoJ in modulating chemoresponsiveness in human tumors. RhoJ siRNA efficiently inhibited RhoJ expression at both the RNA and protein level (Supplementary Fig. S4B). Moreover, RhoJ depletion potently sensitized MNT-1 cells (Fig. 1E) and SK-Mel-28 cells (Supplementary Fig. S3A and S3B) to cisplatin-induced apoptosis as measured by the accumulation of Annexin V–positive cells. In contrast, RhoJ overexpression enhanced melanoma chemoresistance (Fig. 1F), suppressed cisplatin-induced PARP cleavage (Fig. 1G), and also inhibited cisplatin-induced apoptosis (Fig. 1H). Taken together, these results identify RhoJ as a suppressor of DNA damage–induced apoptosis.

RhoJ regulates melanoma chemoresistance by uncoupling ATR from Chk1

While published studies have revealed that Rho and CDC42 signaling pathways regulate melanoma invasion (25), these GTPases were not known to regulate chemoresistance. To determine how RhoJ regulates melanoma chemoresistance, we sought to identify downstream pathways activated by RhoJ and determined how these pathways modulate the DDR. RhoJ is a CDC42 homologue, a class of GTPases that can bind and activate group I Pak kinases that contain Pak autoinhibitory domains (26). We used a group I Pak inhibitor (IPA-3) to determine whether Pak inhibition sensitized melanoma cells to cisplatin-induced apoptosis. IPA-3 and cisplatin acted synergistically to inhibit ATP accumulation in melanoma cells (Fig. 2A). On the other hand, melanocytes were very sensitive to low-dose IPA-3 (Fig. 2B), and addition of cisplatin only slightly

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depletion sensitizes melanoma cells to cisplatin-induced apoptosis. MNT-1 cells were transfected with the indicated siRNAs for 48 hours and incubated in the presence and absence of 30 μmol/L cisplatin for 48 hours. Apoptotic cells were defined as Annexin V–positive, whereas necrotic cells were defined as PI-positive Annexin V–negative population by flow cytometry. F, overexpression of RhoJ promotes melanoma chemoresistance. The relative sensitivity of vector-infected or RhoJ-overexpressing C8161 cells to 30 μmol/L cisplatin for 48 hours was measured using a CellTiter-Glo ATP accumulation assay. **, P < 0.01 versus vector, by the Student t test. Bar represents mean ± SD (left). G, RhoJ overexpression inhibits cisplatin-induced PARP cleavage. C8161 and SKM28 RhoJ-overexpressing or control vector–infected cells were incubated in the presence of the indicated doses of cisplatin for 24 hours. The relative accumulation of cleaved PARP was measured by immunoblotting. H, RhoJ overexpression inhibits cisplatin-induced apoptosis. C8161 RhoJ-overexpressing or control vector–infected cells were incubated in the presence of 60 μg/mL cisplatin for 24 hours. Apoptotic cells were defined as Annexin V–positive, whereas necrotic cells were defined as PI-positive Annexin V–negative by flow cytometry. Cis, cisplatin; CTL, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Vec, vector.
enhanced the inhibitory effect of IPA-3 on ATP accumulation (Fig. 2B). Next, we asked whether group I Pak kinases (PAK1, PAK2, or PAK3) are activated by RhoJ. Using an antibody that can recognize phospho-Ser199/204 PAK1/3 or Ser192/197PAK2, we observed that cisplatin treatment induced the accumulation of a band that corresponds to p-PAK1/3 in both MNT-1 and SK-Mel-28 melanoma cells (Fig. 2C). Cisplatin did not induce the accumulation of a band that corresponds to phospho-Pak2, which migrates at a different molecular weight, indicating that cisplatin activates either PAK1/3. Using antibodies specific for PAK1 and PAK3, we show that Pak3 expression is undetectable in the cells studied (Supplementary Fig. S4C), suggesting that cisplatin activates PAK1. Importantly, cisplatin induced the accumulation of phospho-PAK1 in a RhoJ-dependent manner (Fig. 2C), whereas Pak1 depletion sensitized both SK-Mel-28 and MNT-1 melanoma cells to cisplatin-induced PARP cleavage (Fig. 2D). These results are consistent with published studies that have shown that RhoJ can activate PAK1 (27) and indicate that RhoJ modulates melanoma chemoresponsiveness by activating PAK1. If this is true, then depletion of PAK1 in RhoJ-overexpressing cells should sensitize these cells to cisplatin-induced apoptosis. While RhoJ overexpression induced the phosphorylation of PAK1 and suppressed cisplatin-induced PARP cleavage (Fig. 2E), depletion of PAK1 in the context of RhoJ overexpression inhibited phospho-PAK1 accumulation and restored the level of PARP cleavage observed in vector-expressing cells (Fig. 2E), consistent with the contention that PAK1 is activated by RhoJ to deplete DNA damage–induced death. While we cannot exclude that RhoJ can activate other group I Paks in other melanoma cell lines when they are expressed, our results indicate that RhoJ activates PAK1 to modulate melanoma chemoresponsiveness in the cell lines studied.

**Table 1.** Ras family GTPases potently sensitize melanoma cells to cisplatin

<table>
<thead>
<tr>
<th>Gene</th>
<th>EMBL-EBI family</th>
<th>GO biologic processes</th>
<th>GO molecular function</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHOJ</td>
<td>Ras GTPase; small GTPase, Rho type; Ras</td>
<td>Actin cytoskeleton organization; regulation of cell shape; regulation of small GTPase–mediated signal transduction</td>
<td>GTP binding; GTPase activity</td>
<td>Lower RhoJ expression correlates with sensitivity to epigenetic therapy for sarcoma</td>
</tr>
<tr>
<td>RND2</td>
<td>Ras GTPase; small GTPase, Rho type; Ras</td>
<td>Small GTPase–mediated signal transduction</td>
<td>GTP binding; GTPase activity</td>
<td>Activates RhoA</td>
</tr>
<tr>
<td>ARL4A</td>
<td>Ras GTPase; ADP ribosylation factor; ARF/SAR superfamily</td>
<td>Small GTPase–mediated signal transduction</td>
<td>GTP binding; protein binding</td>
<td>Genetically amplified in melanoma</td>
</tr>
<tr>
<td>PPP1R12C</td>
<td>Protein phosphatase 1, regulatory subunit 12A/B/C, eukaryote</td>
<td>Not available</td>
<td>Not available</td>
<td>Regulates mitosis</td>
</tr>
<tr>
<td>PMS2</td>
<td>DNA mismatch repair protein</td>
<td>Mismatch repair; reciprocal meiotic recombination; somatic hypermutation of immunoglobulin genes</td>
<td>ATP binding; ATPase activity</td>
<td>Repair for damages caused by cisplatin; direct p53 effector; interact with MLH1</td>
</tr>
<tr>
<td>BLM</td>
<td>DNA helicase, ATP-dependent, RecQ type</td>
<td>Double-strand break repair via homologous recombination; G2 phase of mitotic cell cycle; G2–M transition DNA damage checkpoint</td>
<td>ATP binding; bubble DNA binding; DNA strand annealing activity</td>
<td>BLM-deficient cells are more sensitive to cisplatin; interact with MLH1 and p53</td>
</tr>
<tr>
<td>SMARCE1</td>
<td>Other</td>
<td>Chromatin modification; negative regulation of transcription, DNA-dependent; nervous system development</td>
<td>Chromatin binding; DNA binding; N-acetyltransferase activity</td>
<td>Regulates chromatin remodeling during replication</td>
</tr>
</tbody>
</table>

NOTE: GO annotation data and PubMed searches were used to identify critical cancer phenotypes regulated by targets identified in this screen.

Abbreviation: GO, Gene Ontology.
depletion sensitized cells to dacarbazine, an agent that generates DNA mismatches that are known to activate the ATR kinase [ref. 28 (#10926)]. Once activated, ATR phosphorylates multiple different targets, including H2AX and Chk1, which can then mark the sites of stalled replication forks or induce cell-cycle arrest/apoptosis, respectively (17). There is some inherent redundancy in this system, as ATM, a kinase that is induced upon double-strand break formation, can also phosphorylate H2AX when ATR is not active (29). Cisplatin induced similar amounts of p-H2AX accumulation in RhoJ-depleted or control siRNA–treated cells (Supplementary Fig. S5A). RhoJ depletion itself did not induce DNA damage and cisplatin induced a similar amount of DNA damage in RhoJ-depleted or control siRNA–transfected cells (Supplementary Fig. S5B). Taken together, these observations indicate that RhoJ does not inhibit DNA damage from occurring.

Once we had determined that RhoJ does not prevent DNA damage, we sought to determine whether RhoJ/PAK1 modulates the cellular response to DNA damage. Upon DNA damage, ATR localizes to chromatin where it is activated (30). ATR then phosphorylates Chk1 on Ser345, which can initiate cell-cycle arrest or induce apoptosis (30). RhoJ depletion when coupled with cisplatin treatment induced the selective accumulation of Chk1 phosphorylated at its ATR-dependent sites (Fig. 3A), indicating that RhoJ modulates ATR activity. Surprisingly, pChk2, a kinase that is phosphorylated specifically by ATM (28), accumulates in untreated cells but not in cisplatin-treated cells (Fig. 3A), suggesting that RhoJ modulates ATR and not ATM activity. PAK1 depletion or IPA-3 treatment when coupled with cisplatin treatment also induced the accumulation of pChk1 (Fig. 3A), indicating that RhoJ/PAK1 normally suppress Chk1 activation. Finally, RhoJ overexpression suppressed cisplatin-induced accumulation of p-Ser345-Chk1 (Fig. 3B), showing that RhoJ and Pak1 inhibits the ability of ATR to phosphorylate Chk1 (30).

Once we had shown that RhoJ suppresses ATR activation, we next asked whether RhoJ regulates cisplatin-induced apoptosis in an ATR-dependent manner. While RhoJ depletion when coupled with cisplatin induced the accumulation of cleaved PARP, co-depletion of ATR and RhoJ suppressed cisplatin-induced cleaved PARP accumulation in both p53 wild-type (C8161) and p53-mutant (SK-Mel-28) cells (Fig. 3C). Similarly, depletion of RhoJ or ATR alone when coupled with cisplatin treatment induced the accumulation of Annexin V–positive
Figure 3. RhoJ and Pak kinases suppress ATR activation. A, depletion of RhoJ/Pak1 or group I Pak inhibition enhances Chk1 activation. MNT-1 or SK-Mel-28 (SKM28) melanoma cells were transfected with the indicated siRNAs for 48 hours and incubated in the presence of 30 µmol/L cisplatin (RhoJ-depleted cells) or 15 µmol/L cisplatin (Pak1-depleted cells) for 24 hours. p-Ser345-Chk1 (ATR-dependent site), Chk1, p-Thr68-Chk2 (ATM-dependent site), and actin accumulation was measured by immunoblotting. SKM28 cells were also treated with the indicated dose of the group I Pak inhibitor IPA-3 and cisplatin, and p-Ser345-Chk1 accumulation was measured. B, RhoJ suppresses Chk-1 activation upon cisplatin treatment. C8161 and SKM28 cells overexpressing RhoJ or infected with the control vector were incubated in the presence of the indicated doses of cisplatin for 24 hours. The relative accumulation of pChk1 was measured by immunoblotting. C, ATR depletion mitigates the effects of RhoJ depletion on cisplatin-induced PARP cleavage. C8161 and SKM28 melanoma cells were treated with indicated siRNA for 48 hours and then incubated with 30 µmol/L cisplatin for 24 hours. Relative accumulation of cleaved PARP was measured. D, ATR depletion mitigates the effects of RhoJ depletion on cisplatin-induced apoptosis. MNT-1 melanoma cells were transfected with the indicated siRNAs, and apoptosis was quantified by measuring Annexin V staining. E, RhoJ modulates claspin accumulation. MNT-1/SKM28 melanoma cells were transfected with the indicated siRNAs for 72 hours, and relative accumulation of claspin was measured by immunoblotting. C8161 and SKM28 melanoma cells overexpressing RhoJ expressed lower levels of claspin when compared with cells that express a control vector (right). F, RhoJ modulates claspin accumulation via a mechanism involving Pak1. SKM28 cells were transfected with control or Pak1 siRNAs for 48 hours and then treated with or without 30 µmol/L cisplatin for 48 hrs. The relative accumulation of claspin was measured by immunoblotting. G, Pak1 depletion restores RhoJ overexpressing-induced Plk1 accumulation. Vector or RhoJ-overexpressing C8161 cells were treated with control or Pak1 siRNA for 72 hours and analyzed by immunoblotting. Cis, cisplatin; CTL, control; Vec, vector.
cells (Fig. 3D, top), whereas co-depletion of ATR and RhoJ suppressed the cisplatin-induced accumulation of Annexin V–positive cells (Fig. 3D, bottom). Depletion of Chk1 alone was highly toxic, so we were unable to test whether RhoJ modulates chemo-resistance in a Chk1-dependent manner (data not shown). Nonetheless, our observations indicate that RhoJ suppresses the DDR by an ATR-dependent mechanism.

Next, we sought to determine how RhoJ/Pak1 uncouples ATR from Chk1. Claspin, a scaffold that couples ATR to its downstream effector Chk1 (31), can be phosphorylated by the Pak1 target PLK1 (32). Phosphorylated claspin is subsequently degraded by the ubiquitin–proteasome pathway (31). We found that RhoJ overexpression suppressed claspin accumulation, whereas RhoJ depletion stimulated claspin accumulation (Fig. 3E), suggesting that RhoJ modulates ATR signaling by modulating claspin accumulation. Similarly, Pak1 depletion modulated claspin accumulation both in the absence and in the presence of cisplatin (Fig. 3F). RhoJ overexpression induced the accumulation of Plk1, the kinase that phosphorylates claspin and induces its degradation. Pak1 depletion suppressed the accumulation of Plk1 induced by RhoJ overexpression (Fig. 3G), consistent with a role for Pak1 in activating Plk1. Taken together, our results indicate that RhoJ modulates the DDR by suppressing the ability of ATR to phosphorylate its downstream effectors, including Chk1.

Published studies have indicated that Chk1 primarily functions to induce cell-cycle arrest (33), although it can also initiate apoptosis by phosphorylating p53 (34). Depletion of
Figure 5. RhoJ inhibits Sox10 expression in melanoma cells. A, RhoJ is required for Sox10 expression in melanoma cells but not melanocytes. The relative mRNA expression of RhoJ and SOX10 was measured by reverse-transcription quantitative PCR in C8161, SK-Mel-28 (SKM28) melanoma cells, and melanocytes expressing control or 2 different RhoJ shRNAs (left). **P < 0.01 comparing with shCTL, determined by the Student t test. Sox10 protein accumulation in RhoJ-deficient melanocytes and melanoma cells was measured via immunoblotting (right). B, RhoJ modulates the expression of Sox10 in melanoma cells and melanocytes. Relative mRNA expression of RhoJ and Sox10 in RhoJ-overexpressing SKM28 melanoma cells was quantified using reverse-transcription quantitative PCR (left). **P < 0.01 comparing with shCTL, determined by the Student t test. Lysates from C8161, SKM28 melanoma cells, and melanocytes overexpressing RhoJ were subjected to Western blotting with Sox10 and actin antibodies to measure the relative accumulation of RhoJ (right). C, Sox10 expression is modulated by the DDR. MNT-1 and SKM28 cells were treated with 30 μmol/L cisplatin at the indicated ...
RhoJ suppressed proliferation (Fig. 4A, representative images in Supplementary Fig. S5D) and enhanced S-phase arrest (Fig. 4B) in the presence but not in the absence of cisplatin in both p53 wild-type and mutant cells, indicating that RhoJ suppressed Chk1-dependent cell-cycle arrest. As Chk1 can also induce apoptosis by activating p53, we next sought to determine whether RhoJ regulates cisplatin-induced apoptosis in a p53-dependent manner. PAK1 and RhoJ depletion induced the selective accumulation of p53 and p53 phosphorylated at Ser20 (a Chk1-dependent phosphorylation site; ref. 34) in p53 wild-type cells (Fig. 4C). In contrast, PAK1 or RhoJ depletion did not induce the accumulation of p53 (Fig. 4D) or p-Ser20-p53 (data not shown) in p53 mutant SK-Mel-28 melanoma cells. Co-depletion of p53 and RhoJ suppressed cisplatin-induced PARP cleavage (Fig. 4E) and cisplatin-induced Annexin V accumulation in p53 wild-type cells (Fig. 4F). While co-depletion of p53 and RhoJ suppressed cisplatin-induced PARP cleavage in p53-mutant cells (SK-Mel-28; Fig. 4E), it did not inhibit Annexin V accumulation in these cells (Supplementary Fig. S3C). Taken together, these results suggest that RhoJ regulates cisplatin-induced apoptosis in a p53-dependent manner in wild-type cells. In p53-mutant cells, the ability of RhoJ to modulate chemoresponsiveness is only partially dependent on p53, consistent with published studies indicating that mutant p53 can be partially functional (35).

Next, we sought to better understand how RhoJ regulates melanoma chemoresponsiveness in p53-mutant cells. In the absence of functional p53, Chk1 can modulate apoptosis by phosphorylating the p53 homologue p73 (36). Unfortunately, co-depletion of p73 and RhoJ did not inhibit cisplatin-induced Annexin V accumulation (Supplementary Fig. S3C). Still other studies have identified NBS1 as a protein that is a downstream target of ATR (37) that can modulate DNA damage–induced apoptosis (38). Co-depletion of NBS1 and RhoJ did suppress cisplatin-induced apoptosis in p53-mutant cells (Supplementary Fig. S3C). Moreover, cisplatin treatment induced the phosphorylation of NBS1 at its ATR-specific phosphorylation site only in RhoJ siRNA–treated cells (Supplementary Fig. S3D), indicating that RhoJ normally suppresses cisplatin-induced NBS1 phosphorylation. Taken together, these results suggest that RhoJ regulates melanoma chemoresistance by suppressing the ability of ATR to phosphorylate its downstream effectors, resulting in decreased activation of Chk1 and the decreased phosphorylation of Chk1 targets including p53. In p53-mutant cells, RhoJ can suppress cisplatin-induced apoptosis both by inhibiting the activation of hypofunctional p53 and by suppressing the ability of ATR to phosphorylate other effectors, such as NBS1. Interestingly, other targets identified in our screen did not modulate Chk1 activation but did modulate p53 accumulation (Supplementary Fig. S5C), reinforcing the concept that p53 is a central regulator of melanoma chemoresponsiveness. 

RhoJ modulates melanoma chemoresistance by modulating the expression of Sox10

To gain a better appreciation of molecular pathways regulated by RhoJ, we next sought to identify RhoJ-regulated genes and pathways using a microarray-based approach (see dataset, Supplementary Table S3). Gene set enrichment analysis (39) revealed that regulators of neural development were significantly up- or downregulated in cells expressing RhoJ short hairpin RNA (shRNA) when compared with cells expressing control shRNAs (Supplementary Fig. S6A). In particular, chronic RhoJ depletion potently inhibited the expression of Sox10 (>100-fold) as well as Sox10 target genes (Supplementary Fig. S6B). Chronic RhoJ depletion was sufficient to inhibit both Sox10 mRNA/protein accumulation in C8161 and SK-Mel-28 melanoma cells (Fig. 5A). While RhoJ depletion did not inhibit the accumulation of Sox10 mRNA in melanocytes, it did inhibit the accumulation of Sox10 protein in these cells (Fig. 5A). RhoJ overexpression induced the accumulation of Sox10 mRNA and protein in both SK-Mel-28 cells and normal melanocytes (Fig. 5B), indicating that RhoJ regulates the expression of Sox10.

Sox10, a known onconege in melanomas, regulates the survival of neural crest precursor cells before lineage commitment in the developing embryo (40) and is a reliable marker for melanoma tumor–initiating cells (41). Recent studies have determined that Sox10 is required for melanoma formation in both mice and humans and determined that depletion of Sox10 alone is sufficient to induce apoptosis in melanoma cells (42). ATF2, a transcription factor that is activated by ATM/ATR signaling, has recently been shown to suppress Sox10 expression in melanoma cells (43). Depletion of ATR inhibited the phosphorylation of ATF2 at its ATR/ATM target sites while also inducing the accumulation of Sox10 (Fig. 5C), consistent with a role for ATR in modulating ATF2 function.
and repressing Sox10 expression. In contrast, RhoJ or Pak1 depletion or Pak1 inhibition resulted in the accumulation of ATF2 phosphorylated at its ATR target sites (Fig. 5D). Pak1 depletion or inhibition ultimately resulted in the decreased accumulation of Sox10 (Fig. 5E). Sox10 depletion inhibited melanoma cell survival (Supplementary Fig. S6C) and sensitized melanoma cells to cisplatin-induced PARP cleavage (Fig. 5F). In addition, cisplatin treatment inhibited Sox10 accumulation (Fig. 5G), indicating that ATR activation can suppress Sox10 expression. Intriguingly, Sox10 overexpression inhibited cisplatin-induced apoptosis in p53-mutant cells (Fig. 5H), and Sox10 overexpression partially mitigated the impact of Pak inhibitors on cisplatin sensitivity (Fig. 5I). These results indicate that Sox10 expression also promotes melanoma chemoresistance. In summary, our results indicate that RhoJ and Pak1 limit the response to DNA damage while concomitantly promoting cell proliferation.

**Discussion**

In this study, we have used an unbiased genome-wide functional genomics approach to identify RhoJ and its downstream kinase Pak1 as novel regulators of response of the melanoma cell to DNA damage. Cisplatin activates Pak1 in a RhoJ-dependent fashion, indicating that RhoJ activates Pak1 to modulate melanoma chemoresistance. While RhoJ does not inhibit cisplatin-induced DNA damage, it does suppress the phosphorylation of the ATR downstream effectors Chk1 and ATF2. In p53 wild-type cells, RhoJ-induced ATR suppression leads to a functional inactivation of p53. In p53-mutant cells, RhoJ-induced ATR suppression results in the decreased phosphorylation of the ATR target NBS1 and the increased expression of Sox10, which can also suppress drug-induced apoptosis. Importantly, parallel studies have determined that Pak1 depletion or Pak kinase inhibition recapitulated all of the phenotypes observed upon RhoJ depletion, suggesting that RhoJ regulates all of these phenotypes, in part, by activating Pak1. Taken together, these studies identify the suppression of DNA damage sensing, as opposed to the commonly accepted mechanism of apoptosis execution, as a linchpin of chemoresistance in melanoma and nominate RhoJ and Pak1 as optimal therapeutic targets for the rational design of novel synergistic chemotherapeutic regimens.

Melanocytes in the epidermis produce melanin in response to UVB irradiation and transfer this pigment to keratinocytes, protecting them from UV-induced DNA damage (44). To accomplish this, melanocytes must possess mechanisms to resist DNA damage-induced apoptosis and also facilitate the expression of genes that regulate melanin production and survival (45). Of interest, our results suggest that RhoJ and Pak1 limit the response to DNA damage while concomitantly facilitating the expression of central regulators of melanogenesis. RhoJ/Pak1 suppress ATR activation, which can also be induced by UV, allowing the melanocyte to tolerate higher levels of DNA damage than other epidermal cells. ATR suppression in turn leads to the increased expression of Sox10, which can in turn regulate MITF, a transcription factor that controls the expression of multiple genes required for melanin production (46). Elevated expression of Sox10 is observed in melanoma tumors, and Sox10 is required for melanoma formation (42). Similarly, RhoJ is overexpressed in advanced melanomas as compared with primary melanomas (Supplementary Table S3). In addition, melanoma tumors that occur in UV-exposed skin are known to accumulate large numbers of mutations (47), suggesting they have an increased DNA damage tolerance. These observations suggest that in the normal setting, RhoJ/Pak1 activity allows melanocytes to tolerate limited amounts of DNA damage to facilitate the production of melanin in response to UV stress. When this pathway is activated in melanoma, the resulting tumor cells are profoundly resistant to DNA damage agents and have an increased DNA damage tolerance as evidenced by their accumulation of multiple mutations. Taken in context, our results indicate that melanoma cells are intrinsically chemoresistant because they activate cell-autonomous, lineage-selective pathways that blunt the responsiveness of the DNA damage surveillance machinery, thus allowing them to persist in the face of high levels of DNA damage. Development of agents that selectively disable RhoJ/Pak1 pathway activity may therefore represent an opportunity to resensitize melanoma tumors to conventional chemotherapeutic agents.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
RhoJ Regulates Melanoma Chemoresistance

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Acknowledgments

The authors thank Stacie Loftus and Bill Pavan for their suggestions, Keith Hook for his assistance with the RhoJ tumor expression data, and Kyoko Yokomori and Roberta Barunio for their advice about the COMET assay and p53 sequencing.

Grant Support

This work was supported by the NIH (1K08AR056001 to A.K. Ganesan and CA71443 to M.A. White), the UC Cancer Research Coordinating Committee, the American Cancer Society (1215-03-SG-11-128-01-CSM to A.K. Ganesan), Outrun the Sun, Inc., and the Robert E. Welch Foundation (I-1414 to M.A. White). The microscopy studies were supported by a P41-RR01192. This work was also partially supported by UL1 RR033185 from the National Center for Research Resources (NCRR).

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Received February 29, 2012; revised July 27, 2012; accepted August 20, 2012; published OnlineFirst September 12, 2012.

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