A critical role for phosphatase haplodeficiency in the selective suppression of deletion 5q MDS by lenalidomide

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Lenalidomide is the first karyotype-selective therapeutic approved for the treatment of myelodysplastic syndromes (MDS) owing to high rates of erythroid and cytogenetic response in patients with chromosome 5q deletion [del(5q)]. Although haploinsufficiency for the *RPS14* gene and others encoded within the common deleted region (CDR) have been implicated in the pathogenesis of the del(5q) phenotype, the molecular basis of the karyotype specificity of lenalidomide remains unexplained. We focused our analysis on possible haplodeficient enzymatic targets encoded within the CDR that play key roles in cell-cycle regulation. We show that the dual specificity phosphatases, Cdc25C and PP2Acα, which are coregulators of the G_2-M checkpoint, are inhibited by lenalidomide. Gene expression was lower in MDS and acute myeloid leukemia (AML) specimens with del(5q) compared with those with alternate karyotypes. Lenalidomide inhibited phosphatase activity either directly (Cdc25C) or indirectly (PP2A) with corresponding retention of inhibitory phospho-tyrosine residues. Treatment of del(5q) AML cells with lenalidomide induced G_2 arrest and apoptosis, whereas there was no effect in nondel(5q) AML cells. Small interfering RNA (shRNA) suppression of *Cdc25C* and *PP2Acα* gene expression recapitulated del(5q) susceptibility to lenalidomide with induction of G_2 arrest and apoptosis in both U937 and primary nondel(5q) MDS cells. These data establish a role for allelic haplodeficiency of the lenalidomide inhibitable Cdc25C and PP2Acα phosphatases in the selective drug sensitivity of del(5q) MDS.

cancer treatment | myelodysplastic syndromes | drug sensitivity | Cdc25C | PP2A

Myelodysplastic syndrome (MDS) with chromosome 5q deletion [del(5q)] is the only cytogenetically defined MDS category recognized by the World Health Organization (WHO) (1). Interstitial deletion of chromosome 5q is the most common chromosomal abnormality detected in MDS with an overall case prevalence of 10%–15% (2, 3). MDS patients with an isolated chromosome 5q deletion have distinct clinical and pathological features that include a refractory hypoproliferative anemia, dysplastic megakaryocytes with preserved or increased thrombopoiesis, and an indolent clinical course. Deletion mapping of the commonly deleted region (CDR) involved in the so-called '5q-syndrome' identified a 1.5 megabase segment extending between bands 5q31 and 5q32 containing 44 genes (4). Expression of a number of candidate genes implicated in the pathogenesis of the del(5q) phenotype is reduced commensurate with mono-allelic deletion, including the ribosomal processing gene *RPS14*, the secreted protein acidic and rich in cysteine (*SPARC*/*osteonectin*), alpha-catenin (*CTNNA1*), and early growth response gene-1 (*EGR-1*) (5–9). By using a functional RNA interference screen of 41 genes in the CDR, Ebert et al. showed that only inactivation of the *RPS14* gene impaired erythroblast differentiation and viability while preserving megakaryocyte maturation (6). Moreover, lentivirus transduction of *RPS14* cDNA into primary del(5q) marrow cells was sufficient to rescue erythropoiesis.

In December 2005, the United States Food and Drug Administration (FDA) approved lenalidomide (Revlimid; Celgene Corporation) for the treatment of transfusion-dependent, lower-risk patients with del(5q) MDS (10, 11). Lenalidomide is remarkably active in del(5q) MDS, giving rise to transfusion independence with concordant cytogenetic response in more than two-thirds of treated patients. In contrast, in a large multicenter trial involving transfusion-dependent MDS patients without del(5q), only 26% achieved transfusion-independence with infrequent cytogenetic improvement (12). These data and evidence from laboratory investigations indicate that the erythropoietic effects of lenalidomide in MDS are both karyotype dependent and mechanistically distinct (10, 11). Lenalidomide selectively inhibits the in vitro growth of del(5q) MDS progenitors (13), whereas in MDS with alternate karyotypes and normal bone marrow CD34+ cells, lenalidomide and its analogue, pomalidomide, promote erythroid lineage competence and colony-forming capacity (14, 15). The molecular targets of lenalidomide that account for its selective activity in MDS remain undefined. By using gene expression profiling, Pellagatti and colleagues showed that lenalidomide induced expression of the CDR-encoded *SPARC* gene in erythroblasts from both MDS patients with del(5q) and normal marrow donors (13). The sole differentially regulated gene in lenalidomide-treated MDS erythroblasts was *activin A*, whose role, if any, in treatment response is unknown. We previously reported that lenalidomide enhances erythropoietin receptor signaling in nondel(5q) MDS progenitors by inhibiting negative regulatory phosphatase activity (16). We hypothesized that lenalidomide inhibits the activity of 1 or more haplo-deficient phosphatases encoded within the CDR that have key roles in cell survival and proliferation to account for its selective suppression of del(5q) progenitors. Here, we provide evidence that lenalidomide is selectively cytotoxic to del(5q) cells as a result of inhibition of the haplodeficient dual specificity phosphatases, Cdc25C and PP2A.

Results

Lenalidomide Is Selectively Cytotoxic in Del(5q) AML Cells. To determine if lenalidomide is selectively cytotoxic to del(5q) clones, we first evaluated apoptotic response in AML cells with and without

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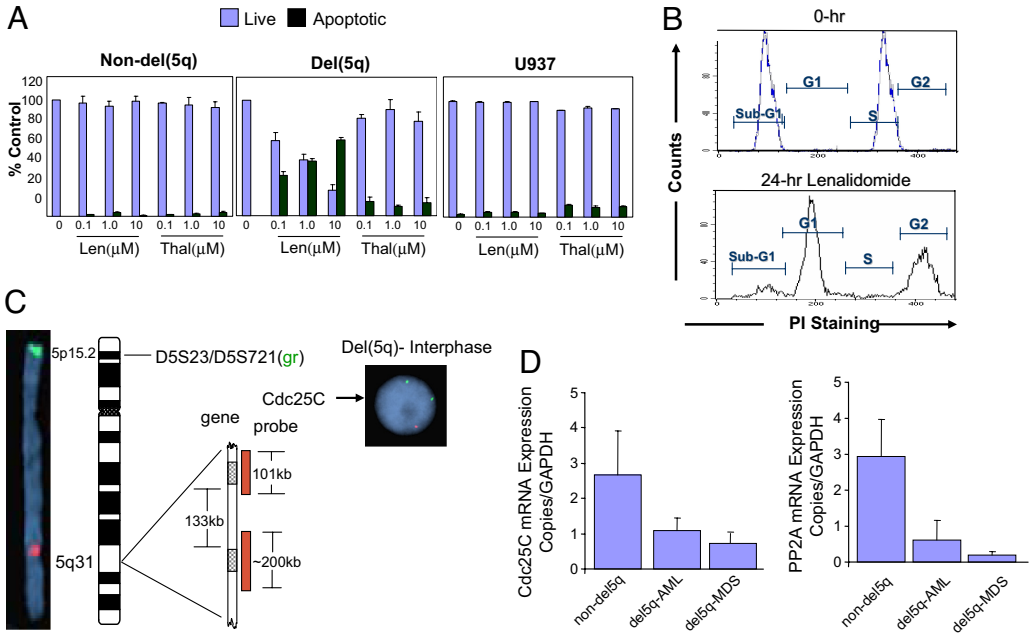

Fig. 1. Apoptosis induction by lenalidomide is specific for 5q-deleted cells (*A*) Cells from AML that evolved from MDS patients with (*Center*) or without (del)5q and U937 cells (*Left* and *Right*) were exposed to lenalidomide, thalidomide, or vehicle (DMSO) at the concentrations indicated for 48 h before apoptosis was assessed by flow cytometry using Annexin-V/ PI staining. Representative results are shown as the mean of the triplicate measurement ± SD from 1 patient. A total of 5 different MDS patients were tested. (*B*) Lenalidomide arrests 5q deleted cells in G_2. Cells were treated with lenalidomide at the concentration of 1 μM for 48 h at 37 °C and stained with propidium Iodide (PI) in BD Stain Buffer (10^6/ml) before analysis on BD FACScan. (*C*) FISH analysis of haplo-deficiency of Cdc25C in (del)5q cells. A normal chromosome 5 showing FISH signals for D5S23/D5S721 (green) and Cdc25C (orange); the ideogram demonstrates the relative gene locations of EGR1 and Cdc25C in 5q31. Probes for EGR1 are commonly used to detect classical 5q deletions. The *Right Inset* illustrates D5S23/D5S721 and Cdc25C signal pattern for classical 5q deletions in interphase nuclei. (*D*) Reduced expression of Cdc25C and PP2Acα in bone marrow cells from patients with (del)5q by Q-PCR. RNA was purified from BM-MNC from patients with or without (del)5q as indicated. Relative expression levels of Cdc25C and PP2Acα were analyzed by Q-PCR to quantitate transcript level. Expression is normalized to the reference gene (GAPDH) and fold changes for Cdc25C and PP2Acα in patients are compared with the average data from non(del)5q cells ($P < 0.001$).

del(5q) (Table S1). Apoptosis was assessed by flow cytometry after annexin-V and propidium iodide staining in myeloblasts from a patient with isolated del(5q) AML that evolved from MDS. Lenalidomide induced a concentration-dependent increase in apoptosis after 48-h exposure in del(5q) AML cells (Fig. 1*A*, *Center*), but had no effect in the nondel(5q) U937 cell line and normal karyotype AML that evolved from MDS (MDS/AML) ($n = 5$, Fig. 1*A Left* and *Right*). Treatment with equimolar concentrations of thalidomide had no effect on cell viability at concentrations up to 10 μM. To determine whether apoptotic response to lenalidomide in del(5q) cells is associated with arrest in a specific phase of the cell cycle, we assessed time-dependent change in cell cycle after lenalidomide exposure. The apoptotic effect of lenalidomide in del(5q) AML cells was associated with G_2 arrest (Fig. 1*B*), whereas in nondel(5q) primary AML and U937 cells there was no significant change in cell cycle after 72 h of drug exposure. These findings indicate that isolated chromosome 5q interstitial deletion is associated with selective sensitivity to lenalidomide characterized by induction of G_2 arrest and apoptotic cell death.

Haplodeficiency of the *Cdc25C and PP2Aca* Phosphatases in Del(5q) MDS. The 1.5 Mb CDR in del(5q) contains 2 dual specificity phosphatases that are complimentary coregulators of the G_2-M checkpoint, i.e., the cell division cycle 25 C (*Cdc25C*) and the catalytic subunit alpha isoform (*PP2Ac*α) and its regulatory subunit B (*beta isoform PR 52*) of the protein phosphatase 2A (PP2A) (17, 18). Cyclin-B/cyclin-dependent kinase-1 (CDK-1, or cdc2) complexes are normally inhibited until G_2-M entry by phosphorylation of tyrosine (Tyr^{15}) and threonine (Thr^{14}) residues within the ATP binding domain of CDK-1(cdc-2). Dephosphorylation by Cdc25C, and to a lesser extent Cdc25A, enables CDK-1 activation and cell-cycle progression. Mitotic entry requires Cdc25C dephosphorylation by PP2A to promote 14–3-3 disassembly and nuclear translocation (19, 20).

We next examined whether the *Cdc25C* gene, which resides at band 5q31.2, is hemizygously deleted in del(5q) patient specimens by interrogating with a custom fluorescent in situ hybridization (FISH) probe. The *Cdc25C* gene probe showed no overlap with the *EGR1* locus on chromosome 5q31, which is ≈133 kb telomeric (Fig. 1*C*). We confirmed *Cdc25C* haplo-deficiency in 46%–78.5% (median, 72%) of interphase nuclei from del(5q) primary bone marrow MDS specimens (Fig. 1*C*, $n = 5$). In contrast, a probe specific for 5p15.2, *D5S23/D5S721*, showed the presence of both copies on the short arm of chromosome 5 in all cells examined, whereas both *Cdc25C* alleles were found in nondel(5q) specimens ($n = 5$) and the U937 cell line. By using real-time quantitative PCR (Q-PCR), we compared the level of gene expression of *Cdc25C* and *PP2Ac*α in del(5q) AML ($n = 2$) and MDS ($n = 3$) to normal karyotype ($n = 5$) MDS primary marrow specimens. Expression level of *Cdc25C* and *PP2Ac*α mRNA was significantly lower in each of the del(5q) specimens tested compared with non5q specimens with a mean 2.5-fold reduction in the specific gene transcripts (Fig. 1 *D*) and ($P <$ 0.001), thereby providing evidence for concordance between gene dosage of *Cdc25C* and *PP2Ac*α and mRNA expression level.

Lenalidomide Inhibits Cdc25C and PP2A Dual Specificity Phosphatase Activity. Having demonstrated reduction in gene expression commensurate with gene dosage, we investigated whether lenalidomide inhibits Cdc25C and PP2A phosphatase activity in intact cells. We first assessed change in phosphorylation of the inhibitory tyrosine substrates for Cdc25C on CDK-1/Cdc2 [Tyr^{15}] and the PP2A substrate regulatory serine on Cdc25C [Ser^{216}] by immunoblot after treatment of U937 cells with lenalidomide or the PP2A selective inhibitor fostriecin as a positive control. Fostriecin is the most selective small molecule protein phosphatase inhibitor identified to date and is a potent inhibitor of PP2A (21). Lenalidomide treatment increased retention of both phospho-Tyr^{15} Cdc2 and phospho-Ser^{216} Cdc25C in a concentration dependent fashion, which was recapitulated by fostriecin exposure, indicating intracellular inhibition of both phosphatases (Fig. 2*A*). To determine whether lenalidomide directly inhibits Cdc25C and PP2A activity, we evaluated phospho-tyrosine peptide hydrolysis using rhu-Cdc25C and rhu-PP2A. We found that lenalidomide is a potent inhibitor of rhu-Cdc25C phosphatase activity with a 50% inhibitory concen-

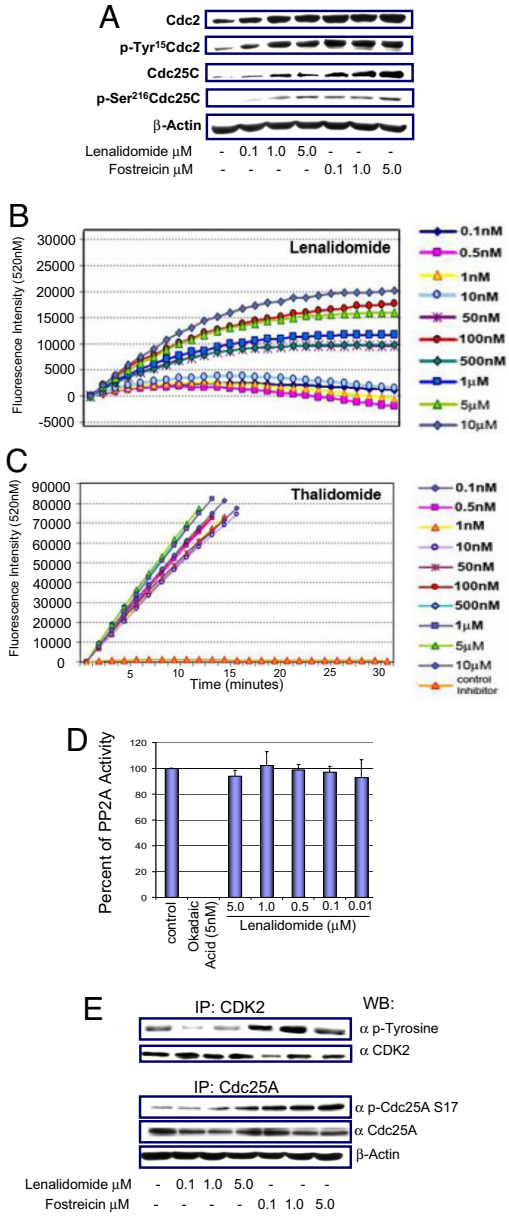

Fig. 2. Lenalidomide promotes the retention of inhibitory phosphates on Cdc2 and Cdc25C. (*A*) U937 cells were treated with vehicle, 0.1, 1, or 5 μM lenalidomide for 24 h. Western blots were performed with antibodies specific for phosphorylated Cdc2 (*p*-Tyr^{15} Cdc2) or phosphorylated Cdc25C (*p*-Ser^{216} Cdc25C). (*B*) Lenalidomide directly inhibits Cdc25C phosphates (PPT) activity in vitro. (*C*) Thalidomide did not display direct inhibition of Cdc25C PPT activity. (*D*) Lenalidomide has no direct inhibitory effect on PP2A PPT activity in vitro. Okadaic acid (a serine/threonine phosphatase inhibitor) was used here as a positive control for PP2A PPT activity. (*E*) Cdc25A dephosphorylation of CDK-2 is inhibited by lenalidomide. U937 cells were either treated with lenalidomide or fostriecin (PP2A inhibitor) at the concentration indicated and then lysed. Immunoprecipitation (IP) was performed with either anti-CDK-2 (*Upper*) or Cdc25A (*Lower*). The samples were separated by SDS PAGE and then Western blotted with antibodies either specific to phosphotyrosine (*p*-tyrosine) or phospho-S17 of CDC25A (*p*-CDC25A S17) as indicated.

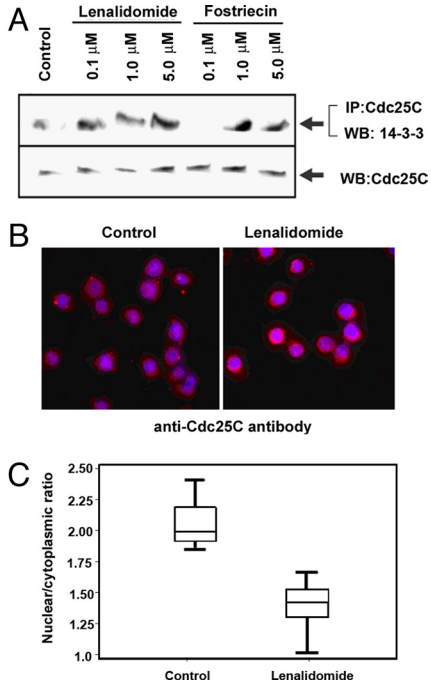

Fig. 3. Lenalidomide promotes Cdc25C binding to 14–3-3 proteins. (*A*) U937 cells treated with the indicated concentrations of lenalidomide or fostriecin for 3 h then lysed. Immunoprecipitation was performed with anti-Cdc25C and Western blotted with antibodies either specific to 14–3-3 or Cdc25C to show the equal loading of protein in each lane. (*B*) Lenalidomide increased Cdc25C cytoplasmic sequestration. U937 cells were treated with or without lenalidomide at the concentration of 1 μM for 3 h. Cytospins were prepared and stained with anti-Cdc25C primary antibody, AlexaFlour-594 secondary, and DAPI (original magnification, 400×). Quantitative analysis of nuclear/cytoplasmic ratio of Cdc25C for lenalidomide-treated cells is significantly lower compared with that of the control ($P < 0.0001$).

tration (IC_{50}) of 10 nM after 30 min of drug exposure, whereas thalidomide had no effect (Fig. 2 *B* and *C*, respectively). Neither compound, however, inhibited rhu-PP2A phosphatase activity (Fig. 2*D*), indicating that intracellular inhibition of PP2A enzymatic activity by lenalidomide is indirect. To determine whether lenalidomide is an isotype-specific inhibitor of Cdc25, we next assessed the effect of drug treatment on Cdc25A enzymatic activity, the principal regulator of CDK-2 and the G_1-S phase checkpoint. Lenalidomide had no inhibitory effect on rhu-Cdc25A, but promoted CDK-2 phospho-tyrosine retention in immunoblots from drug-treated cells (Fig. 2*E Top*). Intracellular inhibition of Cdc25A activity was confirmed by assessment of tyrosine phosphatase activity in Cdc25A immunoprecipitates from lenalidomide-treated U937 cells, with >75% inhibition at concentrations <0.1 μM. Intracellular inhibition of Cdc25A activity was associated with increased phosphorylation of the inhibitory Ser^{17} on Cdc25A that was recapitulated after fostriecin treatment (Fig. 2*E Bottom*). These findings indicate that lenalidomide is a direct inhibitor of the Cdc25C phosphatase, while indirectly inhibiting the activity of PP2A and its G_1-S phase regulatory target, Cdc25A.

Cdc25C activity is controlled by phosphorylation on Ser^{216} to create a consensus binding site for the 14–3-3 binding proteins, which restricts nuclear import of Cdc25C from the cytoplasm (20, 22–25). To determine whether lenalidomide induces retention of phospho-Ser^{216} on Cdc25C and increases binding to 14–3-3 proteins (26), extracts from lenalidomide- and fostriecin-treated cells were subjected to coimmunoprecipitation assays. Treatment with lenalidomide increased the amount of 14–3-3β coimmunoprecipitation with Cdc25C in a concentration-dependent fashion, analogous to the effect of fostriecin (Fig. 3*A*), indicating that accumulation of the Ser^{216} phosphorylated protein is associated with enhanced cytoplasmic complex formation. Altered intracellular trafficking of Cdc25C in response to lenalidomide exposure was examined by immunofluorescence staining of U937 cells with anti-Cdc25C antibody. Cdc25C displayed cytoplasmic retention with reduced nuclear staining after lenalidomide (Fig. 3*B*) or fostriecin treatment.

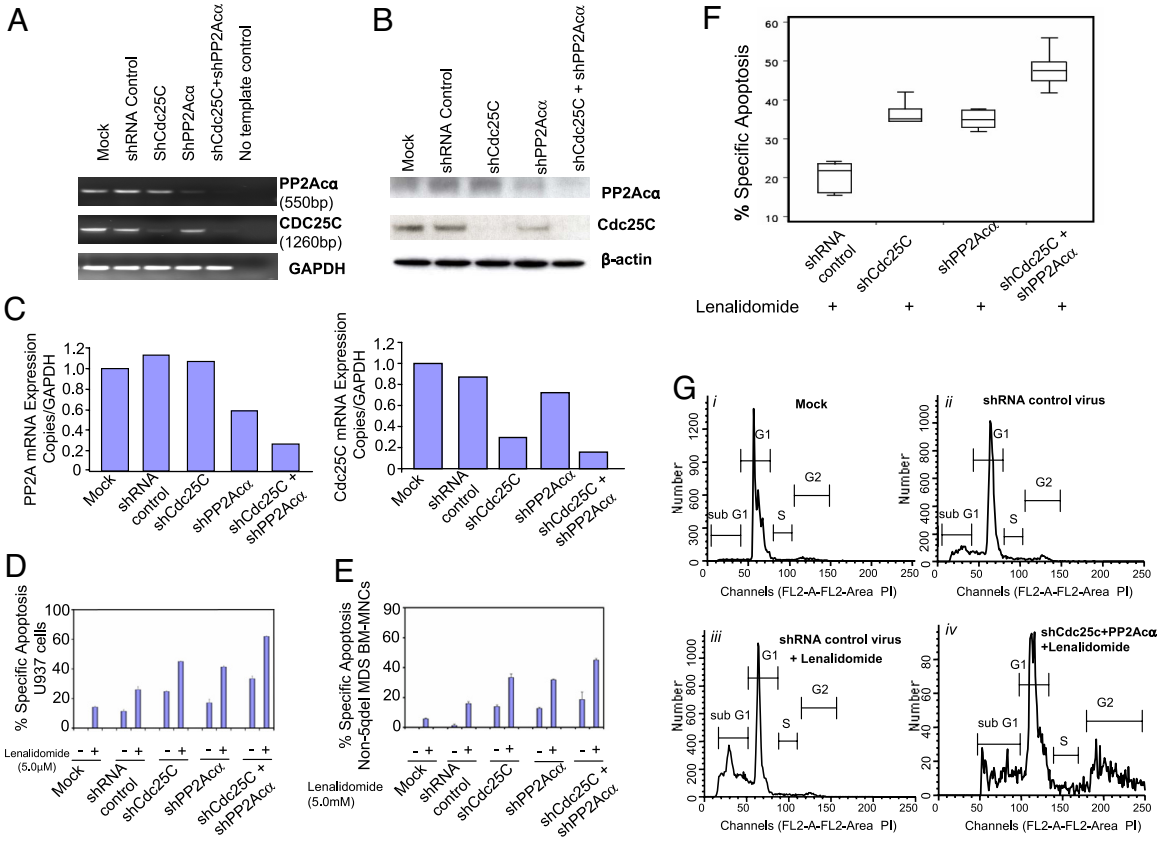

Fig. 4. Dual Cdc25C and PP2Acα gene knockdown increases apoptotic response to lenalidomide-treated cells. (*A–C*) Dual shRNA knockdown of Cdc25C and PP2Acα expression in lentivirus-infected U937 cells. U937 cells were infected with mock (lane 1), lentiviral vectors encoding nontarget shRNA control (lane 2), shCdc25C (lane 3), shPP2Acα (lane 4), shCdc25C and PP2Acα (lane 5), and negative for PCR (lane 6). After 48 h of incubation, the cells were harvested for gene expression analysis using RT-PCR (*A*), Western blot analysis (*B*), and Q-PCR analysis of the suppression effect of shRNA specific for PP2Acα (*Top*) and Cdc25C (*Middle*) (*C*). (*D* and *E*) Dual Cdc25C and PP2Acα gene knockdown increases apoptotic response to lenalidomide in U937 cells and BM-MNC from non(del)5q MDS patients. The U937 cells (*D*) or BM-MNC (*E*) were infected with lentiviral vectors containing various constructs as indicated for 48 h and then treated with or without lenalidomide at the concentration of 5 μM for additional 48 h before analyzed for apoptosis. (*F*) The difference between Cdc25C and PP2Acα double-knockdown plus lenalidomide treatment and shRNA control plus lenalidomide was statistically significant ($P < 0.001$) in 5 MDS patients with a normal karyotype. (*G*) Cell cycle analysis of BM-MNC from MDS patients with a normal karyotype after Cdc25C/PP2Acα double-knockdown plus lenalidomide treatment. A representative result is shown from 1 patient. A total of 5 different MDS patients were tested.

Quantitative image analysis shows that nuclear to cytoplasmic ratio is much lower in lenalidomide-treated cells than control cells ($1.40 \pm 0.18/2.07 \pm 0.17$), indicating that Cdc25C-Ser^{216} phosphorylation and associated 14–3-3 complex formation promote cytoplasmic sequestration of Cdc25C (Fig. 3*C*).

Dual Knockdown of *Cdc25C* and *PP2Acα* genes Promotes G_2 Arrest and Apoptotic Response to Lenalidomide. To investigate the relationship between *Cdc25C* and the *PP2Acα* gene expression level and apoptotic response to lenalidomide, we used a recombinant lentiviral-based gene delivery system carrying short hairpin RNAs (shRNA) to reduce both *Cdc25C* and *PP2Acα* gene expression. Recombinant lentiviruses have proven to be an effective tool to deliver genes into human cells (6, 27). Lentiviral infection in U937 cells significantly reduced corresponding gene transcript levels after 48 h as depicted in Fig. 4. The selectivity and magnitude of *Cdc25C* and *PP2Acα* suppression in response to shCdc25C and shPP2A in U937 cells is shown by RT-PCR and Western blot analysis in Fig. 4 *A* and *B*, respectively. Quantification of Western blot data using densitometry indicated that shRNA specifically reduced *Cdc25C* expression by 83.2% and *PP2Acα* expression by 74.3%, respectively. The inhibition of *Cdc25C* and PP2Acα transcripts was also confirmed by Q-PCR analysis (Fig. 4*C*). By using these genetically modified U937 cells to mimic the haplo-deficiency of both targets in del(5q) cells, we assessed apoptotic response after lenalidomide treatment. As shown in Fig. 4*D*, U937 cells infected with shRNA control virus were marginally sensitive to the apoptotic effects of lenalidomide, demonstrating 25% specific apoptosis after drug treatment. Introduction of shCdc25C and shPP2Acα into U937 cells resulted in an additive increase in apoptosis to 61% (difference of 36% compared with shRNA control-infected cells), suggesting that reduced expression of these gene products promotes sensitivity to the apoptotic effects of lenalidomide. These findings were replicated in 5 primary patient specimens by introduction of shCdc25C and shPP2Acα into BM-MNC cells from nondel(5q) MDS specimens (Fig. 4*E*). The mock-infected and control shRNA-treated BM-MNC cells displayed relative resistance to lenalidomide treatment, while shCdc25C or shPP2Acα single knockdown cells showed only an increase of ≈30% in the apoptotic fraction. However, we observed just 16% apoptosis in our shRNA control versus 45% in shCdc25C and shPP2Acα double knockdown after lenalidomide treatment. Thus, on dual reduction of *Cdc25C* and *PP2Acα* gene expression by lentivirus shRNA, lenalidomide induced 3-fold greater apoptosis compared with the control shRNA-treated cells. The difference between double knockdown plus lenalidomide treatment and shRNA control plus lenalidomide was statistically significant in 5 MDS patients with a normal karyotype, as shown in Fig. 4*F* ($P < 0.001$). These findings support the importance of gene dosage or expression level of both genes in

conferring sensitivity to the apoptotic effects of lenalidomide in MDS specimens. Cell-cycle analysis using BM-MNC cells from 5 nondel(5q) MDS patients showed that lenalidomide treatment increased G_2 arrest in dual *Cdc25C* and *PP2Acα* shRNA infected cells compared with control-virus-infected cells (Fig. 4*G*), analogous to our findings in the del(5q) AML specimen.

Discussion

Lenalidomide is the first karyotype-selective therapeutic approved by the FDA for the treatment of MDS based on its activity in patients with chromosome 5q deletion (7, 8). Lenalidomide has broad biological effects ranging from inhibition of angiogenesis to enhancement of tumor-specific immunity (28, 29). Nevertheless, these effects are not karyotype dependent and are unlikely to account for the selective suppression of del(5q) MDS clones. The high frequency of erythroid response to lenalidomide in del(5q) MDS is directly linked to suppression of the malignant clone, evidenced by the complete concordance between hematologic and cytogenetic response (10, 11). Our findings indicate that lenalidomide has a concentration-dependent cytotoxic effect in del(5q) progenitors characterized by preferential cell-cycle arrest in G_2 accompanied by induction of programmed cell death at low micromolar concentrations achieved in vivo (Fig. 1) (30). These findings are consistent with those reported by Pellagatti et al. showing that lenalidomide selectively suppressed the in vitro growth of del(5q) MDS erythroblasts (13). In our studies, we show that shRNA suppression of *Cdc25C* and *PP2Acα* gene expression in U937 and nondel(5q) MDS bone marrow cells to levels commensurate with mono-allelic deletion replicated lenalidomide sensitivity to G_2 arrest and apoptosis in del(5q) AML cells (Fig. 4), indicating a critical role for dosage of these genes in drug sensitivity. Interestingly, lenalidomide triggered apoptosis in 10%–20% of nontransfected and nontarget shRNA transfected controls, consistent with its broad antitumor effects unrelated to del(5q). Lenalidomide up-regulates the expression of *SPARC*, which has been implicated in the antitumor effects of lenalidomide in lymphoid malignancies, and therefore, may cooperate with haplodeficient phosphatase inhibition to enhance apoptotic sensitivity in del(5q) progenitors (13, 31). Moreover, RNAi-mediated knockdown of either the *PP2A* structural A subunit, the catalytic C subunit, or a variable targeting-regulatory B subunit in *Drosophila* destabilizes the hetero-trimeric holoenzyme and diminishes viability, suggesting that haplodeficiency per se for 2 of these subunits as occurs in del(5q) MDS may contribute to the impaired survival capacity of erythroid progenitors and the enhanced susceptibility to the proapoptotic effects of lenalidomide (32). Together, these findings support a model in which reduced gene expression as a result of allelic haplodeficiency in del(5q) MDS promotes increased susceptibility to G_2-M arrest after lenalidomide exposure that leads to selective clonal suppression. Phosphatases are important enzymatic targets for drug design. Nevertheless, despite extensive research, there are currently no marketed protein tyrosine phosphatase inhibitors, a fact that stands in stark contrast to the array of kinase inhibitors. Our findings indicate that lenalidomide may represent the first in an emerging class of therapeutics targeting human phosphatases.

Previous studies have shown that lenalidomide has direct antiproliferative effects in a variety of tumor cell lines. In myeloma and lymphoma cells, lenalidomide preferentially arrests cells in G_1 (33, 34). Our findings that lenalidomide indirectly inhibits Cdc25A, the principal regulator of the Cdk2 complex and G_1–S phase transition, provides a rationale for this antiproliferative effect. Moreover, given the secondary role of Cdc25A in the control of the G_2-M transition, concurrent inhibition of Cdc25A and the haplodeficient Cdc25C and PP2Acα phosphatases may reinforce G_2 arrest of del(5q) MDS progenitors by lenalidomide. Additionally, Kuramitsu et al. recently showed that abnormal ribosomal biogenesis arising from allelic deficiency of either the *RPS19* gene implicated in the pathogenesis of Diamond-Blackfan anemia or *RPS14* impairs erythroid proliferation by activating p53 and arresting progenitors in G_0 (35). Allelic haplodeficiency of *RPS14* therefore may account for the erythroid hypoplasia characteristic of del(5q) MDS and perhaps further increase susceptibility to lenalidomide induced cell-cycle arrest. Moreover, our findings suggest that reduced gene expression arising from either *Cdc25C* and *PP2Acα* allelic haplodeficiency or other molecular mechanisms such as chromosome 5 monosomy, methylation silencing or mutation may promote similar sensitivity to lenalidomide. These data suggest that compensatory changes in these genes or related Cdc25 isotypes may be involved in the emergence of resistance to lenalidomide treatment that merits further investigation. Finally, based on these data, loss of heterozygosity resulting in haplodeficiency of drug-able targets involved in cell survival may represent a strategy for development of tumor-selective therapies in other malignancies.

Materials and Methods

Patients and Preparation of BM-MNC from MDS Patients. MDS untreated patients ($n = 15$) were recruited from the Malignant Hematology clinic at the H. Lee Moffitt Cancer Center and Research Institute. Diagnoses were confirmed by pathology and classified in accordance with WHO criteria (Table S1). After obtaining written informed consent, the bone marrow cells were collected in heparinized tubes from each patient. Informed consents were signed by all patients to allow the use of their cells for these experiments. Bone marrow mononuclear cells (BM-MNC) were isolated from patients and normal donors by Ficoll-Hypaque gradient centrifugation, as described in ref. 36.

Cell Culture. U937, a human leukemia monocytic cell line was purchased from ATCC and cultured in RPMI MEDIUM 1640 supplemented with 10% FBS and penicillin-streptomycin. HEK–293T cells were purchased from ATCC and were maintained in DMEM (DMEM) supplemented with 10% calf serum and 100 μg/mL of penicillin-streptomycin.

RNA Isolation and Reverse Transcription Reaction. Total RNA was purified from BM-MNC cells either from AML or MDS patients with or without (del)5q using TRIzol-Reagent according to the manufacturer's instructions (Life Technologies). The purity and concentration were measured by spectrophotometer. Reverse transcription (RT) reactions were performed using iScriptTM cDNA Synthesis kit (BIO-RAD). cDNA was synthesized by adding 1 μg of total RNA, 5x iScript Reaction Mix (4 μL), and 1 μL of iScript Reverse Transcriptase to a total volume of 20 μL. The reaction was incubated at 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 5 min. (See *SI Materials and Methods* for additional details.)

Fluorescence in Situ Hybridization (FISH). FISH was done at the Cytogenetics Laboratory Mayo Clinic (Rochester, MN) according to detailed methods as described in a previous study (36, 37). The Cdc25C FISH probe was created after first obtaining BAC RP11–256P1 from CHORI. DNA from the BAC cultures was isolated and tested by PCR to confirm the presence of Cdc25C DNA. The target DNA was subsequently labeled with fluorescent SpectrumOrange dUTP using nick translation. D5S23/D5S721 served as a control probe for a normal chromosome 5 FISH signals (green).

Analysis of mRNA Expression by Real-Time Quantitative-PCR (Q-PCR). Q-PCR reactions were performed by means of iQ SYBR Green Supermix of Bio-Rad. Each reaction (25 μL) contained 12.5 μL of iQ SYBR green supermix, 0.25 μL of forward primer (20 μM), 0.25 μL of reverse primer (20 μM), 11 μL of RNase-free water, and 1.0 μL of cDNA. The following cycles were performed 1 × 3 min at 95 °C, 40 amplification cycles (15 s 95 °C, 60 s 56 °C), 1 × 1 min 95 °C, 1 × 1 min 55 °C and a melting curve (80 × 10 s 55 °C with an increase of 0.5 °C per 10 s). A negative control without cDNA template was run with every assay. The optimal melting point of dsDNA I and the efficiency of the reaction were optimized beforehand. Transcript copy number per individual was calculated by normalization to GAPDH expression. The relative level of gene expression for each patient was calculated by normalization to the average expression level observed in 5 controls.

Cdc25C and PP2A Phosphatase Assay. The phosphatase assay was performed as outlined in the assay kit instructions (CycLex Protein Phosphatase Fluorometric Assay Kit (MBL International). (See *SI Materials and Methods* for additional details.)

Preparation of Cdc25C, PP2Acα RNAi and Lentivirus. Nucleotide sequences for short hairpin RNA (shRNA) were described and designed for ShRNA-Cdc25C as follows: sense strand, 5′-GAAGAGAATAATCATCGTGTT -3′, and antisense strand, 5′-GAAGAGAATAATCATCGTGTT-3′; sequences for shRNA-PP2Acα were de-

signed as follows: sense strand, 5′-TGGAACTT GACGATACTCTAA-3′, and antisense strand, 5′-TGGAACTTGACGATACTCTAA-3′.

A scrimbord RNAi sequence was used as a nonspecific control. Oligonucleotides were designed that incorporated these sequences within a short hairpin structure, using the stem loop sequence 5′-CTCGAG-3′, which were then cloned into lentiviral plasmids (pLKO.1-puro purchased from Sigma). Lentiviral particles were generated by transfection of lentiviral plasmids and packaging mix (purchased from Sigma) into HEK-293-T cells using lipofectamine 2000 reagent (Invitrogen). Supernatant containing viral particles were harvested between 36 and 72 h. The supernatant was purified and used for Cdc25C and PP2A knocking down experiments. Nontarget shRNA were used as negative control, and lentiviral vectors containing GFP were used to evaluate the infection rate. For lentiviral infection, 0.5×10^6/ml of U937 cells were incubated with recombinant lentiviruses at MOI = 1:5 in the presence of 8 μg/ml of polybrene for 48 h before treatment with lenalidomide.

Apoptosis Assays and Cell-Cycle Analysis. U937 at a concentration of 2.0×10^5 cells per well or BM-BNC at a concentration of 5.0 [times[10^5 cells per well from patient cells were seeded in a 12-well plate. The cells were infected with lentiviral particles containing GFP, nontarget shRNA (negative control), shRNA-Cdc25C, and/or shRNA-PP2Acα. After 48-h incubation at 37 °C, the cells were either untreated or treated with 5 μM of lenalidomide for an additional 48 h. The plates were incubated for 4 d and analyzed for apoptosis using the Annexin V apoptosis kit (BD Pharmagen). Each well was resuspended in 1× binding buffer at a concentration of 1.0×10^6 cells per milliliter and stained with Annexin-FITC and 7-amino-actinomycin D 7-AAD or propidium iodide (PI; Sigma Aldrich) as indicated in the figure legend. All samples were analyzed by flow cytometry using a FACScan within 30 min of staining. For cell-cycle analysis, cells were treated with lenalidomide at the concentration of 1 μM for 24 h at 37 °C, then washed and resuspended in BD Stain Buffer (10^6/ml). Cold ethanol (3 ml) was added while vortexing, and the cells were then incubated overnight at 4 °C and washed 2 times in stain buffer. One milliter of PI staining solution (3.8 mM Na citrate, 50 μg/ml PI in PBS) was added to each cell pellet along with 50 μL of RNase A solution (10 μg/ml). Cell suspensions were incubated for 4 h at 4 °C before analysis on BD FACScan (BD BioSciences).

Western Blot Analyses and Immunoprecipitation. After 48-h infection with lentivirus containing each specific shRNA, U937 cells were pelleted rapidly in a microcentrifuge at 4 °C and solubilized by incubation at 4 °C for 30 min in 1% Nonidet P-40, 10 mM Tris, 140 mM NaCl, 0.1 mM PMSF, 10 mM iodoacetamide, 50 mM NaF, 1 mM EDTA, 0.4 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml pepsatin, and 10 μg/ml aprotinin. Cell lysates were centrifuged at 12,000 × *g* for 15 min to remove nuclei and cell debris. The protein concentration of the soluble extracts was determined by using the Bio-Rad (Bradford) protein assay. For Western blots, 50 μg of protein (per lane) were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to Immobilon membranes, and reacted with either anti-Cdc25C or PP2A. Proteins were detected with the enhanced chemiluminescence detection system (ECL, Amersham).

Immunostaining. U937 cells were starved overnight and incubated with lenalidomide in the presence of serum for 3 h at the concentration as indicated. Cells were centrifuged onto microscope slides and fixed with methanol/acetone (3:1) for 20 min. An anti-Cdc25C antibody (1:200 dilution) and secondary goat anti-mouse Ig AlexaFluor (Sigma) was used to visualize the translocation of Cdc25C. Nuclei were stained with DAPI. Immunofluorescence was detected using a Leitz Orthoplan 2 microscope and images were captured by a charge-coupled device (CCD) camera with the Smart Capture Program (Vysis). On each slide, 100 cells were counted for Cdc25C cytoplasmic retention. Nonspecific binding with secondary antibody alone was not detected. Immunoflorescent images were analyzed using Image Pro Plus 6.2 (Mediacybernetics Inc.). Briefly, nuclear image masks were generated using the DAPI image and subtracted from the AlexaFlour-594 image, leaving the cytoplasmic signal. The resulting image was masked and then subtracted from the AlexFlour-594 image, leaving only the nuclear signal. Mean florescence density was recorded for each fraction, and the nuclear to cytoplasmic mean density ± SD was reported.

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