Multidrug Resistance Decreases with Mutations of Melanosomal Regulatory Genes

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Abstract

Whereas resistance to chemotherapy has long impeded effective treatment of metastatic melanoma, the mechanistic basis of this resistance remains unknown. One possible mechanism of drug resistance is alteration of intracellular drug distribution either by drug efflux or sequestration into intracellular organelles. Melanomas, as well as primary melanocytes from which they arise, have intracellular organelles, called melanosomes, wherein the synthesis and storage of the pigment melanin takes place. In this study, comparisons of congenic cells with and without functional molecules regulating melanosome formation show that sensitivity to the chemotherapeutic agent cis-diaminedichloroplatinum II (cis-platin) significantly increases with the mutation of genes regulating melanosome formation, concomitant disruption of melanosome morphology, and loss of mature melanosomes. Absence of the melanosomal structural protein gp100/Pmel17 causes increased cis-platin sensitivity. Independent mutations in three separate genes that regulate melanosome biogenesis (Dtnbp1, Pldn, Vps33a) also result in increased cis-platin sensitivity. In addition, a mutation of the gene encoding the integral melanosomal protein tyrosinase, resulting in aberrant melanosome formation, also causes increased cis-platin sensitivity. Furthermore, sensitivity to agents in other chemotherapeutic classes (e.g., vinblastine and etoposide) also increased with the mutation of Pldn. In contrast, a mutation in another melanosomal regulatory gene, Hps1, minimally affects melanosome biogenesis, preserves the formation of mature melanosomes, and has no effect on cis-platin or vinblastine response. Together, these data provide the first direct evidence that melanosomal regulatory genes influence drug sensitivity and that the presence of mature melanosomes likely contributes to melanoma resistance to therapy. [Cancer Res 2009;69(3):992–9]

Introduction

The treatment of metastatic melanoma has been hampered by broad resistance of tumor cells to therapy. This is in contrast to many other cancers that are amenable to treatment with agents such as cis-diaminedichloroplatinum II (cDDP; cis-platin), which is effective against most common cancers and is used in first or second line combination therapy for melanoma with some response (1).[3] However, currently, no standard therapeutic regimen provides any clinical prolongation of patient survival rate (2–4), and the mechanism of melanoma drug resistance, which limits effective therapy, is not understood.

The major cytotoxic effect of cDDP is thought to be mediated by translocation of the drug to the nucleus, followed by formation of platinum-DNA adducts. This DNA damage triggers signaling cascades leading to long-term growth arrest and mitotic catastrophe and to apoptosis of the cell (5). Whereas many possible mechanisms of drug resistance have been proposed, no cohesive picture has emerged. One proposed mechanism is the decrease of the accessibility of cis-platin to target molecules, such as DNA, by decreasing intracytoplasmic and intranuclear accumulation of cis-platin, which could occur through a number of ways, including transporter-mediated drug efflux from cells (6) or via sequestration into intracellular organelles (7–9).

Melanosomes are unique intracellular organelles found in melanomas and melanocytes, in which the pigment melanin is synthesized and stored before regulated secretion. Melanosomes undergo a well-characterized series of morphologically recognizable maturational stages during formation (10, 11). Stage I is characterized by intralumenal vesicles; stage II develops an elliptic shape and intralumenal longitudinal fibrils; stage III has evidence of pigment deposition along the fibrils; stage IV melanosomes are filled with pigment, obscuring the fibrils completely (see Fig. 1C). Regulation of melanosome formation is influenced by proteins that direct protein traffic to the melanosome, such as the Hermansky-Pudlak syndrome (HPS) proteins (12); structural melanosomal proteins, such as gp100/Pmel17 (13–15); and melanosomal integral membrane proteins, such as tyrosinase (16). Melanosome structure is impaired by mutations in genes defective in HPS (17, 18), and it has been shown that mutations in gp100/Pmel17 also disrupt melanosome biogenesis (15) and that defects in tyrosinase cause melanosome biogenesis to be blocked (16).

Recently, it was shown that an epithelioid tumor lacking melanosomes was susceptible to cDDP compared with a melanoma with melanosomes and that a melanoma with immature melanosomes was more susceptible to cDDP compared with melanomas with mature melanosomes. In the nonmelanoma cells, cDDP accumulated in the nucleus, whereas in melanoma cells, it was found in melanosomes, with very little in the nucleus (19). The sequestration of cDDP into melanosomes had the effect of diverting cDDP from accumulating in the nucleus, likely preventing interaction with target DNA. Although this previous study showed an association between cDDP resistance in melanomas and sequestration of the drug into melanosomes, a direct demonstration of the dependence of cDDP resistance on the presence of melanosomes was lacking.

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[3] National Comprehensive Cancer Network. Clinical practice guidelines in oncology: Melanoma, v.1. http://www.nccn.org 2008.

Here, we show, by using three systems of congenic cells (human melanoma cells with and without gp100/Pmel17 and primary melanocytes derived from mouse models of HPS or having defective tyrosinase, all of which have impaired melanosome biogenesis), that mutation of genes regulating melanosome formation significantly increases the susceptibility of cells to cDDP and that these genes play a fundamental role in influencing cell response to chemotherapy. This work is the first direct evidence that genes functioning along the pathway of melanosome biogenesis potentially provide novel targets for adjuvant melanoma therapy to modulate drug sensitivity.

Materials and Methods

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay. Cells were plated at 200 to 5,000 per well in a 96-well flat-bottomed plate and incubated for 24 to 48 h; then medium was removed, and cDDP (Sigma) or plain medium was added. After 3 to 6 d, medium with drug was removed and 10 μL/100 μL medium of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as supplied by the kit manufacturer [American Type Culture Collection (ATCC)], were added. Plates were incubated for an additional 6 h, and then 100 μL of detergent reagent supplied by the kit were added. After an overnight incubation at room temperature in the dark, absorbance was recorded at 540 nm. Cell number was determined using a standard curve. Percentage of cells proliferating was calculated with the following equation: % cell proliferation = $C_{PT} / C_0 \times 100$, wherein C_0 = cell number without drug treatment and C_{PT} = cell number post–drug treatment. Each graph shown is representative of at least three separate experiments.

Cell culture. All cell culture reagents were purchased from Invitrogen, unless otherwise noted. MNT-1 human melanoma cells (gift of Dr. Vincent Hearing) were cultured in advanced DMEM [+4,500 mg/L D-glucose, nonessential amino acids (NEAA), 110 mg/L sodium pyruvate], supplemented with AIM-V (10%), fetal bovine serum (FBS; 20%), glutamine (2 mmol/L), penicillin-streptomycin (100 units/mL), and fungizone (2.5 μg/mL). SK-MEL-24 human melanoma cells (ATCC) were cultured in EMEM (Lonza Aalkersville, Inc.; +Earle's buffered saline solution, NEAA, 110 mg/L sodium pyruvate) supplemented with FBS (15%) and glutamine (2 mmol/L). HeLa cells (ATCC) were cultured in EMEM (+EBSS, +NEAA, +sodium pyruvate) supplemented with FBS (10%) and glutamine (2 mmol/L). MelFB human melanoma cells (gift of Dr. Rejean Lapointe) were cultured in RPMI 1640 supplemented with FBS (10%), glutamine (2 mmol/L), penicillin-streptomycin (100 units/mL), and gentamicin (10 μg/mL). B16 murine melanoma cells (ATCC) were cultured in DMEM supplemented with 10% FBS and glutamine. Primary melanocytes were established in cell culture from skin tissue of neonatal mice using a method modified from a protocol to culture human keratinocytes (20). Mice were maintained in an American Association for the Accreditation of Laboratory Animal Care–accredited facility, and all protocols were approved by the San Francisco VA Institutional Animal Care and Use Committee. Neonatal mice not older than 3 d were decapitated, and the bodies were washed twice in 10% Hibiclens (Stuart Pharmaceuticals) in water. The specimens were then washed thrice in PBS containing 50 μg/mL Fungizone [University of California-San Francisco (UCSF) Cell Culture Facility] and penicillin G (2,000 units/mL)/streptomycin (2,000 μg/mL; UCSF Cell Culture Facility). The dorsal back skin was isolated with forceps and then washed thrice in the same concentration of penicillin/streptomycin/Fungizone in PBS and once with a lower concentration [penicillin (500 units/mL)/streptomycin (500 μg/mL)/Fungizone (10 μg/mL)]. The samples were then incubated in HBSS (UCSF Cell Culture Facility) with dispase (BD Biosciences; 2,500 caseinolytic units) and gentamicin (500 μg/mL; Cellgro Mediatech) overnight at 4°C. The epidermis was separated from the dermis with forceps and incubated in 0.05% trpysin/EDTA (Life Technologies Invitrogen) for 10 to 12 min at 37°C. The trypsin was neutralized by adding twice the volume of HBSS with 5% FBS (Hyclone). The cells were pelleted by centrifugation, and trypsin was removed by aspiration. The cells were

plated at three to four specimens per T185-cm^2 flask (Nunc) in 154 medium with 0.07 mmol/L Ca^{2+} and human keratinocyte growth supplement (Cascade Biologics) to allow a keratinocyte feeder layer to grow. The medium was changed after 3 to 4 d to 254 with human melanocyte growth supplement (Cascade Biologics). The cells were fed every 2 to 3 d for 10 to 11 d and then passed to remove any remaining keratinocytes. The cells were maintained at 37°C and 5% CO$_2$.

Electron microscopy. Cells were scraped from culture dishes, pelleted, and fixed for 15 min in modified Karnovsky's fixative [0.1 mol/L cacodylate/2% paraformaldehyde/2% gluteraldehyde/0.06% calcium chloride (pH 7.4)]. The pellets were then carefully dislodged and turned over to allow penetration of fixative from all sides for another 30 min, followed by a postfixation in reduced osmium [2% osmium tetroxide/1.5% potassium ferrocyanide/0.1 mol/L cacodylate buffer (pH 7.4)]. Cell pellets were washed thrice in double-distilled water and postfixed in 2% aqueous uranyl acetate for 1 h at 4°C. Finally, samples were dehydrated in graded ethanol and embedded in Epon resin. Sixty-nanometer sections were collected on formvar-coated copper grids (Ted Pella, Inc.) and counterstained with saturated uranyl acetate in 70% methanol and lead citrate. Samples were visualized with a Zeiss 10C/CR electron microscope. All reagents were purchased at Electron Microscopy Sciences, except for calcium chloride and potassium ferrocyanide (Sigma), as well as ethanol and methanol (Fisher Scientific).

Results

The presence of mature (versus immature) melanosomes is associated with increased resistance to cDDP. First, we sought to confirm the findings that cDDP resistance is associated with the presence of melanosomes. When the HeLa neoplastic cell line lacking melanosomes was treated with cDDP, it was found to be more susceptible to the cDDP effect (Fig. 1A) compared with the MNT-1 melanoma cell line, which is well-characterized to have all four stages of melanosome biogenesis (ref. 21; also Fig. 1B). Next, it was confirmed that the state of maturity of intracellular melanosomes affected the sensitivity to cDDP of a melanoma cell line. SK-MEL-24 human melanoma cells only have immature melanosomes (Fig. 1B). When incubated with cDDP, SK-MEL-24 cells were more susceptible to the drug effect compared with MNT-1 cells, as shown in Fig. 1D and E.

These studies confirm the correlation of the presence of mature melanosomes with cDDP resistance, as previously reported (19). However, these comparisons by us and others (19) are of melanoma and nonmelanoma tumors from unrelated patients with differing genetic backgrounds and, thus, do not provide direct evidence for the dependence of cDDP resistance on the presence of melanosomes because the effects of background genes cannot be discounted. To address this issue, we next compared the cDDP sensitivity of parental cells (with mature melanosomes) with that of congenic mutant cells (with disrupted melanosome biogenesis caused by a single-gene mutation) to establish that increased cDDP sensitivity is specifically attributable to mutation in a single gene affecting melanosome biogenesis.

Absence of the melanosomal structural protein gp100/Pmel17 results in increased cDDP sensitivity. The molecule gp100/Pmel17 is known to play a critical role in melanosome biogenesis (22), and in the absence of gp100/Pmel17, melanosome biogenesis is impaired (13, 15). Therefore, the effect of the presence and absence of gp100/Pmel17 in congenic human melanoma cells on cDDP resistance was tested, with the prediction that the lack of gp100/Pmel17 would cause increased cDDP sensitivity. The melanoma MelFB lost gp100/Pmel17 expression through immunoselection. The congenic cell line MelFB + gp100/Pmel17 was

derived from MelFB by the transduction of gp100/Pmel17 into MelFB; another congenic cell line MelFB − gp100/Pmel17 was generated by transducing MelFB with a control vector encoding the green fluorescent protein (23). Figure 2 shows that absence of gp100/Pmel17 results in an increase in cDDP sensitivity compared with in the presence of gp100/Pmel17, providing the first direct evidence that disruption of the process of normal melanosome biogenesis increases sensitivity to cDDP.

We then sought further direct evidence in another congenic system, mouse models of diseases caused by genes that disrupt melanosome biogenesis; all of these murine strains share the common genetic background of the parental C57BL/6 mouse strain.

Mouse melanoma cells with mature melanosomes are resistant to cDDP. First, it was determined whether mouse melanoma cells with mature melanosomes are resistant to cDDP in a similar manner to human melanoma cells. B16 is a mouse melanoma cell line with melanosomes morphologically similar to those in the human MNT-1 melanoma; B16 is derived from the C57BL/6 mouse, as are the HPS mice whose melanocytes were subsequently examined (data shown below; refs. 17, 18). Figure 3 shows that the B16 mouse melanoma cells are more similar to the human melanoma MNT-1 in resistance to cDDP than to the human melanoma SK-MEL-24, as expected, correlating with the presence of more mature melanosomes in both B16 and MNT-1 (versus immature melanosomes in SK-MEL-24). This result indicated that the mechanism for cDDP resistance may be similar in human and mouse cells and that mouse models of defective melanosome biogenesis are likely useful and applicable models to investigate the basis for cDDP resistance.
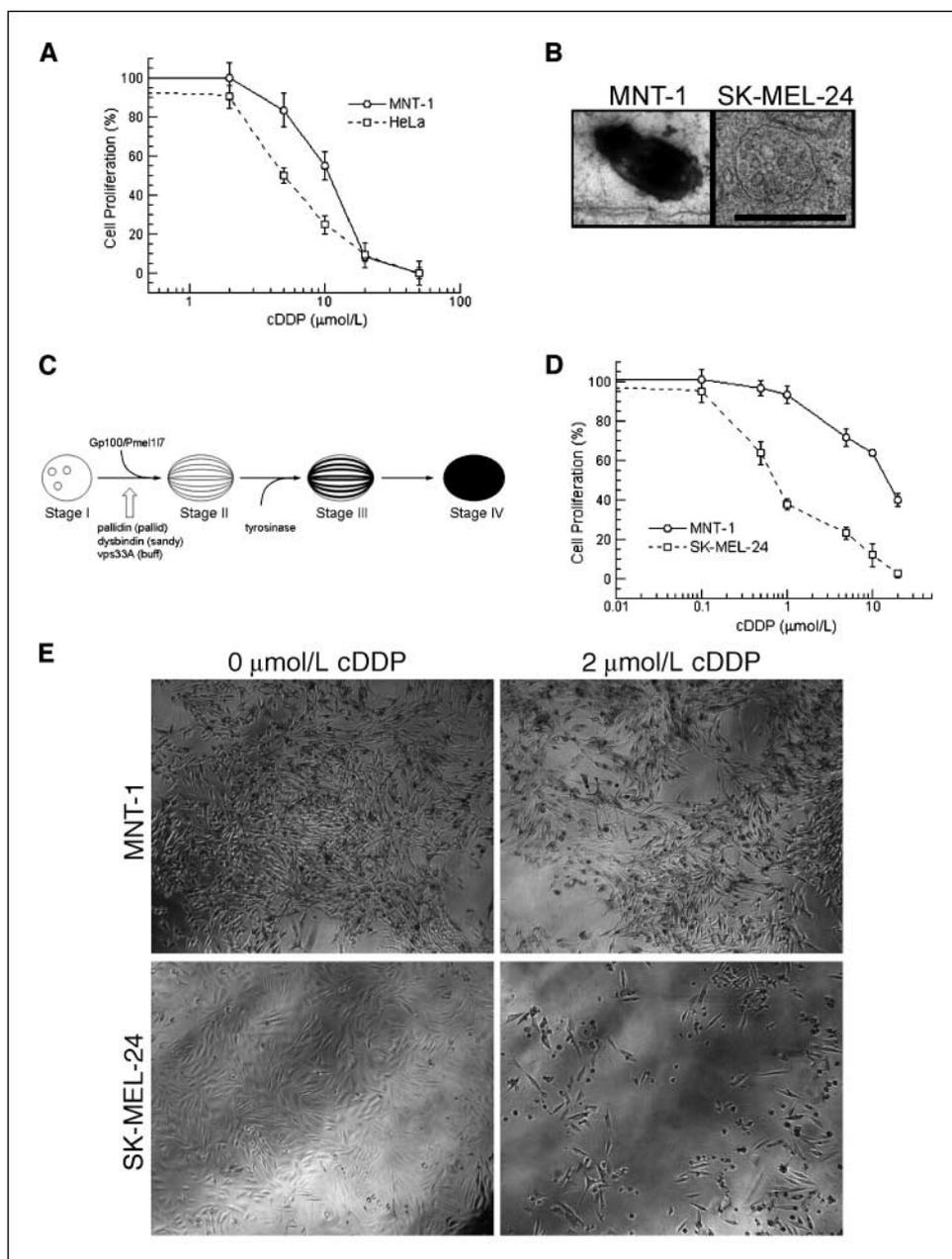
Figure 1. The presence of mature melanosomes correlates with increased resistance to cDDP. *A,* human melanoma MNT-1 (with melanosomes) and nonmelanoma human HeLa tumor cells (without melanosomes) were plated in triplicate into 96-well plates and then treated with or without the indicated concentrations of cDDP for 3 d. Cell proliferation was measured using MTT cell proliferation assay (44–46). Percentage of cell proliferation was calculated as indicated under Materials and Methods. *B,* representative melanosomes from MNT-1 (stage IV) and SK-MEL-24 (stage I) melanoma cells. *Scale bar,* 1.0 μm. *C,* melanosome maturation occurs via four morphologically distinct stages. Transition from stages I to II depends on the function of the structural protein gp100/Pmel17 for formation of longitudinal fibrils. Maturation also depends on the function of HPS proteins pallidin, dysbindin, and vps33a, which regulate protein targeting to the melanosome. Transition from stages II to III depends on the rate-limiting enzyme tyrosinase, which functions in pigment synthesis and contributes to melanosome structure. Mouse strains defective in the respective HPS proteins are noted in parentheses. *D,* cells were plated as in *A* and treated with cDDP for 6 d. Cell proliferation was measured using the MTT assay. *E,* cells were incubated with cDDP for 6 d before being photographed. Beginning at 1 μmol/L cDDP, SK-MEL-24 cells are more severely affected compared with MNT-1 cells; shown is the marked effect of 2 μmol/L cDDP on SK-MEL-24 compared with minimal effect on MNT-1. Note that MNT-1 cells are highly pigmented compared with SK-MEL-24 cells and that the dark appearing cells in the image of SK-MEL-24 cells treated with 2 μmol/L cDDP are nonviable cells.
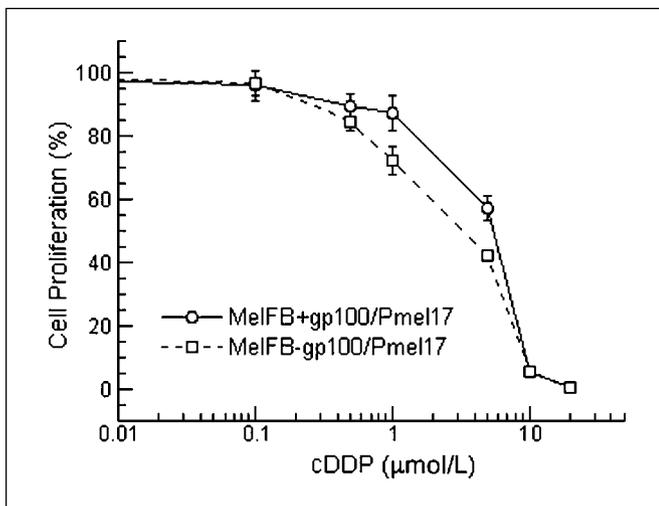
Figure 2. Absence of melanosomal structural protein Pmel17 results in a decreased cDDP resistance. MelFB − gp100/Pmel17 human melanoma cells lack gp100/Pmel17 expression and are more sensitive to cDDP compared with the congenic cell line MelFB + gp100/Pmel17, in which gp100/Pmel17 has been transduced. Cells were plated and treated as in Fig. 1*A*.

Mutations in HPS genes that significantly disrupt melanosome biogenesis result in increased cDDP sensitivity. Mutations in HPS genes have been shown to cause quantifiable and characteristic blocks along various points of the pathway of melanosome biogenesis (17, 18). Thus, it was decided to test if separate and independent mutations in HPS genes, which disrupt melanosome biogenesis, also result in increased cDDP sensitivity compared with control cells with intact melanosome biogenesis. Because the melanocytes tested were derived from mouse strains that are all congenic, due to sharing of the C57BL/6 genetic background, the cDDP sensitivity effect can confidently be attributed to specific gene mutations. The mouse gene mutations noted below are all autosomal recessive; each defective gene has been sequenced, and the mutations were identified (12).

The mouse strain sandy (sdy/sdy) has a mutation in the *Dtnbp1* gene, encoding the dysbindin protein (24). The pallid mouse strain (pa/pa) has a mutation in the *Pldn* gene encoding the pallidin protein (25). Both pallidin and dysbindin are subunits of the BLOC-1 multiprotein complex, which associates with the cytoplasmic face of endosomal membranes. Whereas the exact function of both proteins remains unknown, defects in either one lead to mistrafficking of selected proteins normally targeted to the melanosome (24, 26–29), and this results in melanocytes with significantly impaired melanosome biogenesis (refs. 17, 18; see also Fig. 4*A*). Pallid melanocytes with defective melanosomes incubated with cDDP showed increased drug sensitivity compared with congenic C57BL/6 melanocytes with normal stage IV melanosomes. Melanocytes from sandy mice, also with defective melanosomes, similarly showed increased cDDP sensitivity when compared with C57BL/6 melanocytes (Fig. 4*B*).

The buff mouse strain (buf/buf) has a mutation in the *Vps33a* gene, which is implicated in protein trafficking to the melanosome and lysosome, an intracellular organelle closely related to the melanosome (30, 31).[4] Melanocytes from buff mice also have

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[4] T. Nguyen and M.L. Wei, unpublished observation.

seriously impaired melanosome biogenesis (ref. 18; see also Fig. 4*A*) and show increased drug sensitivity when incubated with cDDP (Fig. 4*C*).

In contrast, our previously published data showed that, in melanocytes from the HPS pale ear mouse strain (ep/ep), stage IV melanosomes are formed but at a slower rate than that seen in the parental congenic C57BL/6 strain (17). Thus, although the rate of melanosome biogenesis is decreased, stage IV melanosomes are present and functional. Cells from pale ear mice were tested for cDDP sensitivity. Figure 4*A* and *D* shows that ep/ep melanocytes have melanosomes with the same stage IV morphology as parental congenic C57BL/6 cells and also have the same resistance to cDDP as do the C57BL/6 cells, consistent with the presence of mature melanosomes conferring resistance to cDDP.

Defect in the melanosomal integral membrane protein tyrosinase disrupts melanosome biogenesis and results in increased cDDP sensitivity. We next sought to determine if defects in a third type of molecule, an integral membrane protein known to influence melanosome formation, would also affect cDDP resistance. Tyrosinase is the rate-limiting enzyme for melanin synthesis and a transmembrane protein localized in the melanosome limiting membrane. Without functional tyrosinase, melanosome morphology is disrupted (16) and a disproportionate number of stage I melanosomes are observed compared with in congenic C576BL/6 melanocytes (Fig. 5*A*). Melanocytes from the tyrosinase mutant mouse C57BL/6J-*Tyr^{c-2J}* were tested for cDDP sensitivity. Figure 5*B* shows that mutation of the tyrosinase gene results in an increase in cDDP sensitivity.

The above data establish that mutations in five separate and independent genes that regulate melanosome formation in three different congenic systems result in increased sensitivity to cDDP together with disruption of melanosome formation. Altogether, these data are the first to show a functional role for melanosomal regulatory genes in cDDP resistance.

Mutation of the melanosomal regulatory gene *Pldn* results in increased sensitivity to vinblastine and etoposide. Finally, we asked if the increased sensitivity to cDDP could be generalized to other chemotherapeutic agents with different modes of cytotoxicity. We tested two other agents as representatives of two other
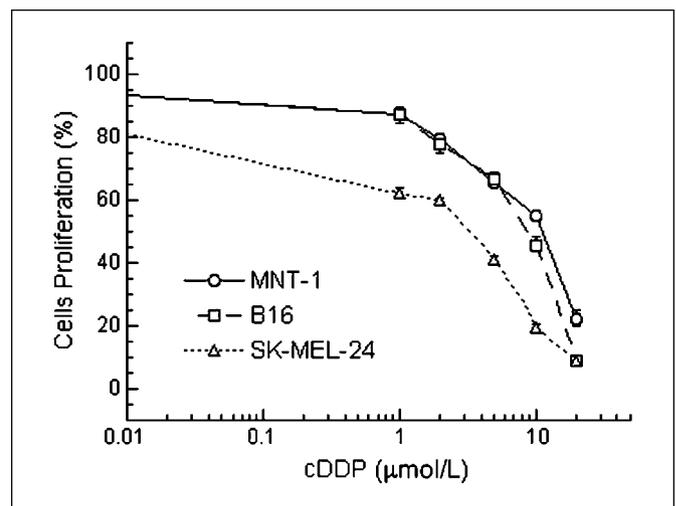
Figure 3. Mouse melanoma cell line B16 is similar to human MNT-1 melanoma cells in having mature melanosomes and in response to cDDP. B16 cells are more resistant to cDDP effects than human SK-MEL-24 cells, which lack mature melanosomes. Cells were plated and treated as in Fig. 1.
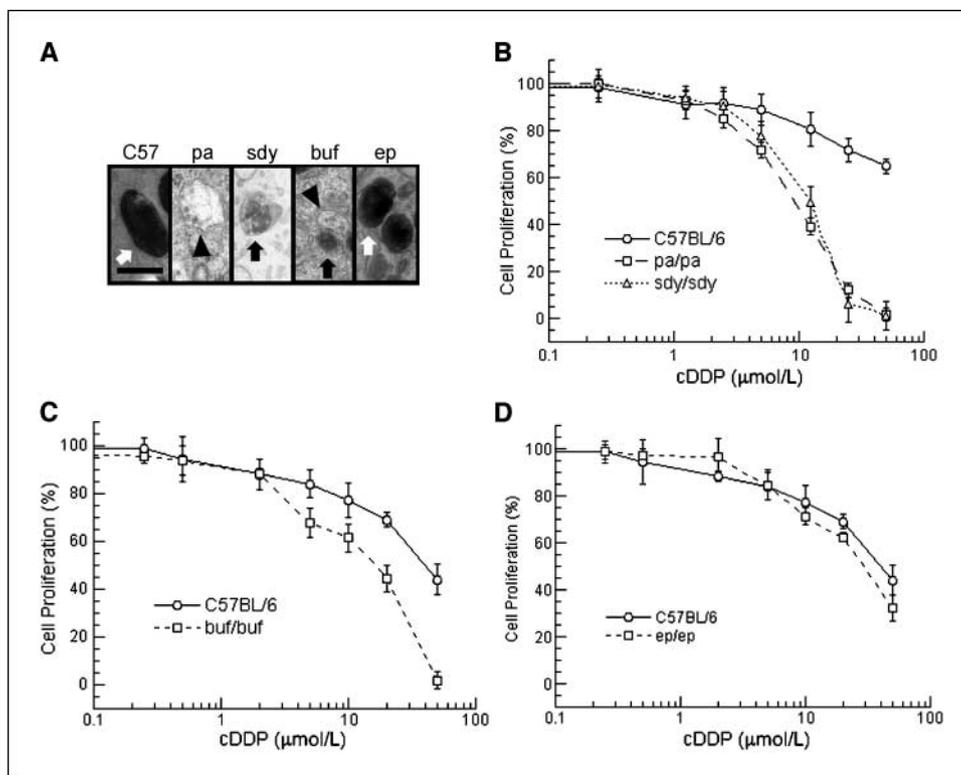
Figure 4. Mutations in HPS genes which significantly disrupt melanosome biogenesis result in increased cDDP sensitivity. *A,* representative melanosomes from HPS mutant mice pallid (*pa*), sandy (*sdy*), buff (*buf*), pale ear (*ep*), and congenic parental wild-type mouse C57BL/6 (*C57*). Melanosome biogenesis is disrupted in pallid, sandy, buff mice with concomitant loss of formation of normal stage IV melanosomes; melanosomes from buff mice are also significantly smaller. Melanocytes from pale ear have stage IV melanosomes, similar to that seen in the congenic parental C57BL/6 strain. *Black arrowheads,* stage I melanosomes; *white arrows,* stage IV melanosome; *black arrows,* aberrantly pigmented organelles. *Scale bar,* 0.5 μm. *B-D,* melanocytes were plated in triplicate into 96-well plates and then treated with or without the indicated concentrations of cDDP for 4 d. Cell proliferation was measured using the MTT assay. Percentage of cell proliferation was calculated, as indicated under Materials and Methods. Note that these are primary melanocytes with a lower proliferative index than melanoma cells and, so, are overall more resistant to cDDP compared with melanoma cells.

classes of drugs: vinblastine, a microtubule poison, and etoposide, which targets topoisomerase. We found that mutation of the HPS *Pldn* gene also increased sensitivity to vinblastine, whereas mutation to the *Hps1/ep* gene, which preserves the formation of mature melanosomes, has no effect on the response to vinblastine. Increased sensitivity was also observed when cells were incubated with etoposide (Fig. 6). Thus, mutations of melanosomal regulatory genes that disrupt melanosome formation increase cell sensitivity to representatives of at least three classes of chemotherapeutic agents, whereas mutation of a regulatory gene that does not affect mature melanosome formation has no effect on drug sensitivity.

Discussion

These data show that genes regulating the formation of mature melanosomes can modulate drug resistance. HeLa cancer cells without melanosomes are more sensitive to cDDP compared with human melanoma cells with mature melanosomes, and melanoma cells with immature melanosomes show increased sensitivity to cDDP compared with melanoma cells with mature melanosomes. These findings confirm a recent report that previously showed similar results (19). Furthermore, our comparisons of congenic sets of cells show that independent mutations in three separate genes that regulate protein trafficking to melanosomes, *Dtnbp1, Pldn*, and *Vps33a*, result in disruption of melanosome biogenesis and concurrent increased drug sensitivity. Absence of the melanosomal structural protein gp100/Pmel17 also results in increased cDDP sensitivity. And finally, mutation in the gene encoding the melanosomal integral membrane protein tyrosinase results in disruption of melanosome biogenesis, as well as increased cDDP sensitivity. Altogether, the data clearly establish that sensitivity to the chemotherapeutic agents cDDP, vinblastine, and etoposide is

enhanced when the function of regulators of melanosome biogenesis is disrupted.

These results suggest that the presence of mature melanosomes confers and is causally related to increased resistance to cDDP, vinblastine, and etoposide. How might this effect be mediated? It was previously shown that cDDP can be sequestered into melanosomes, shifting intracellular distribution away from the nucleus and decreasing drug accessibility to target DNA (19), and it is possible that melanosomal accumulation of cDDP is mediated by transporter molecules, several of which have been localized to the melanosome by proteomic methods (32–34). It is also possible that cDDP is directly internalized from the extracellular milieu into melanosomes via endocytosis or phagocytosis, thus bypassing a cytoplasm to nucleus route. Supporting this is data showing that platinum compounds can be internalized via phagocytosis and that cells with impaired endocytosis have decreased intracellular accumulation of cDDP (35, 36). Melanosome sequestration may act as a resistance modulator for other compounds, in addition to cDDP, through other drug-specific mechanisms. It has been previously shown that the pigment melanin found in mature, but not in immature, melanosomes can bind to chemotherapeutic agents such as daunorubicin and doxorubicin, thus decreasing *in vitro* drug activity; this binding effect was not found for cDDP (37).

Disrupting melanosome biogenesis could affect the above mechanisms in several ways. Defects in the HPS proteins, which influence protein trafficking to the melanosome, could impair transporter molecule targeting or the targeting of other molecules essential for the resistance function to the melanosome. Lack of the functional gp100/Pmel17 molecule or the integral membrane protein tyrosinase could affect the stability of the resulting immature melanosomes, leading to decreased numbers of melanosomes available to sequester drug. Also, both gp100/Pmel17 and
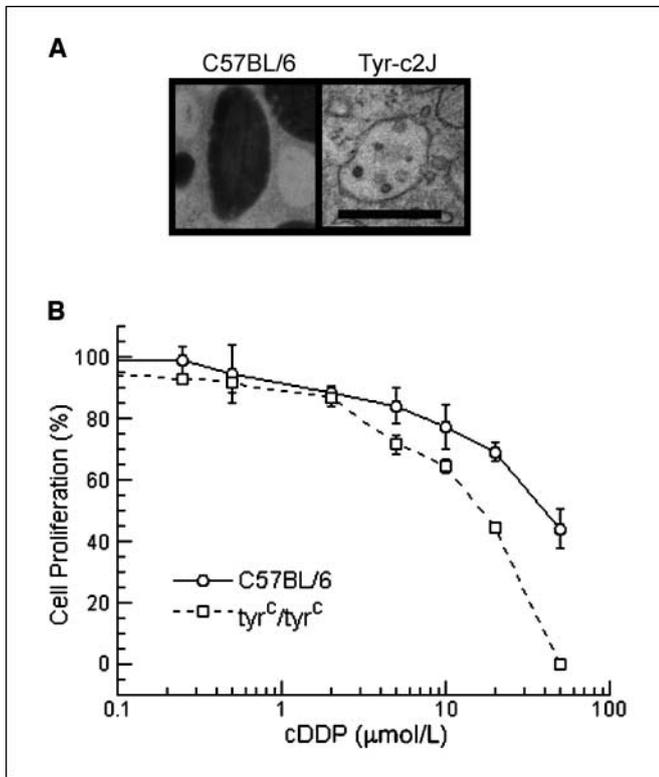
Figure 5. Mutation in tyrosinase disrupts melanosome biogenesis and results in increased cDDP sensitivity. *A,* representative melanosomes from melanocytes derived from C57BL/6J-*Tyr^c-2J* mice (*TyrG291T*) and congenic C57BL/6 mice. *Scale bar,* 0.5 μm. *B,* cells were plated and treated as in Fig. 4. Note that these are primary melanocytes with a lower proliferative index than melanoma cells and, so, are overall more resistant to cDDP compared with melanoma cells.

tyrosinase are known to form complexes with other molecules targeted to the melanosome (e.g., MART-1 and Tyrp1, respectively; refs. 38, 39), so it is possible that the lack of gp100/Pmel17 or tyrosinase affects targeting or function of these and other molecules, contributing to the decreased resistance phenotype.

The mechanism of cDDP cytotoxicity is thought to be multifactorial and probably hinges on platination of DNA and/or protein molecules. Sequestration into melanosomes would be a mechanism by which a drug could be isolated from all cytoplasmic and nuclear targets and could explain melanoma resistance to a broad spectrum of cytotoxic drugs with differing modes of action on varying target molecules. Sequestered drug could be stored, detoxified by glutathione (40), and/or exocytosed and released into the extracellular milieu (9). Supporting this last possibility is evidence showing that, when cultured melanomas were exposed to cDDP, an increased number of melanosomes were exocytosed and secreted into the media (19).

Although we show here that the regulators of melanosome formation influence drug sensitivity, we do not eliminate the possibility that these regulators could mediate drug response via mechanisms alternative to or in addition to melanosome function. It is possible that melanosomal genes have regulatory functions in addition to regulating melanosome formation. For example, they may regulate the formation of other organelles, such as autophagosomes, which might play a role in melanoma chemoresistance. Alternatively, these genes may play a role in cell cycle checkpoint response or apoptotic response. The exact mechanism(s) underly-

ing the chemoresistance mediated by these genes remains to be further investigated.

One question that arises with respect to tumor drug resistance is the potential role of tumor-initiating cells or stem cells. Melanoma stem cells might *a priori* thought to be lacking melanosomes and, thus, would be predicted to be relatively susceptible to drug therapy; this prediction is seemingly contradicted by the stout resistance of clinical tumors to treatment. However, it has been shown that when melanoma cells are incubated with *cis*-platin, an increased number of melanosomes is induced, and evidence suggests that other agents, such as vinblastine, etoposide, and daunorubicin, cause a similar up-regulation (19, 41, 42). Furthermore, a notable side effect of a variety of chemotherapy agents used to treat patients with nonmelanoma cancers is a generalized
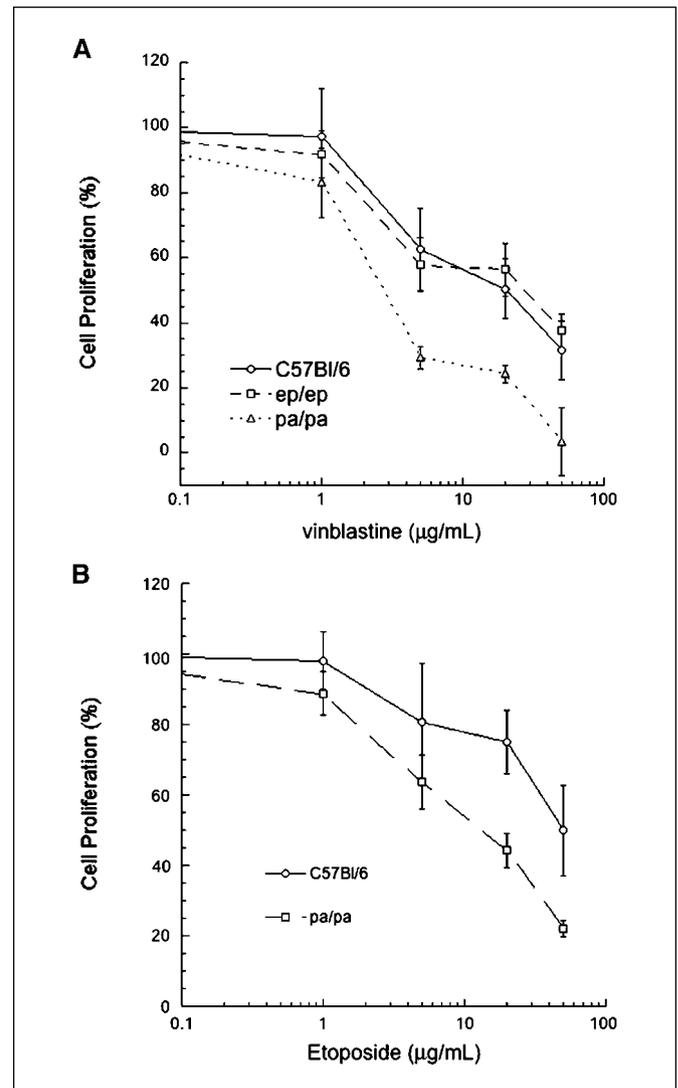
Figure 6. Mutation of HPS gene *Pldn* increases sensitivity to vinblastine and etoposide. *A* and *B,* cells were treated as in Fig. 4 with the indicated drug. Cell proliferation was measured using the MTT assay. Percentage of cell proliferation was calculated, as indicated under Materials and Methods. Note that mutation of the *HPS1/ep* gene, which preserves mature melanosome formation, does not affect vinblastine sensitivity, in contrast to a mutation in *Pldn*, which significantly disrupts melanosome morphology (see Fig. 4*A*) and increases sensitivity to both vinblastine and etoposide. *pa/pa,* pallid melanocytes; *ep/ep,* pale ear melanocytes.

or localized increase in skin pigmentation (43), suggesting that up-regulation of melanosomes or melanin may be a general stress/toxin response. Thus, one mechanism whereby melanoma stem cells could acquire resistance when exposed to chemotherapy could be via the up-regulation of melanosome biogenesis.

Our findings reported here have potentially significant implications for clinical practice. One prediction is that clinically amelanotic melanomas (melanomas lacking pigment), which likely do not have fully mature melanosomes, might be more susceptible to combination chemotherapy, which includes *cis*-platin, vinblastine, and/or etoposide, compared with melanotic melanomas. Current clinical practice does not distinguish between melanotic and amelanotic tumors, and both are assumed to be equally resistant to therapy. Based on our *in vitro* findings, it is predicted that some patients with amelanotic melanomas, an easily identified subpopulation, could potentially be amenable to drug treatment with readily available regimens. Current efforts are under way to examine this issue and test the drug sensitivities of melanotic versus amelanotic tumors.

It is interesting to note that the *in vitro* results indicate that disruption of melanosome formation preserves the difference in drug sensitivity between primary melanocytes and melanoma cells, suggesting that the cDDP toxicity effects of disruptors will be more profound in melanoma tumors versus primary melanocytes, an important aspect for developing clinical applications. Also, whereas it is crucial to do comparisons of congenic cells to unambiguously assign gene influence, it is also important from a clinical standpoint that our data from genetically disparate melanomas

(Figs. 1*D* and 2) suggest that the melanosome functions as a resistance modulator regardless of patient genetic background and that modulating biogenesis could potentially be effective adjuvant therapy for many, if not all, melanoma patients.

In summary, we have shown that genes regulating melanosome formation modulate *cis*-platin, vinblastine, and etoposide sensitivity. This suggests the possibility of targeting the pathway of melanosome biogenesis for therapeutic purposes to modify tumor drug resistance. These genes contribute functionally to the multidrug resistance capacity of melanomas; thus, targeted pharmacologic manipulation may serve to increase melanoma sensitivity to a number of agents with differing modes of activity and has the potential to significantly increase the efficacy of existing melanoma treatments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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