

**SEC61 β CONTROLS SENSITIVITY TO THE PLATINUM CHEMOTHERAPEUTIC AGENTS
THROUGH MODULATION OF COPPER-TRANSPORTING ATPASE ATP7A ***

Paolo B. Abada, Christopher A. Larson, Gerald Manorek, Preston Adams, and Stephen B. Howell

Moores Cancer Center and Department of Medicine

University of California, San Diego, La Jolla, California 92093

Running Title: Control of platinum drug sensitivity by Sec61 β

Address correspondence to : Paolo Abada, MD, Moores UCSD Cancer Center, 3855 Health Sciences Drive, Mail Code 0819, La Jolla, CA 92093-0819; Tel. (858) 822-1110; Fax (858) 822-1111; email: pabada@ucsd.edu

Text pages: 35

Number of tables: 1

Number of figures: 9

References: 55

Number of words

Abstract: 196

Introduction: 744

Discussion : 1488

The abbreviations used are: BCS, bathocuproine disulphonate; cDDP, cisplatin; Cu, copper; ICP-MS, inductively coupled plasma mass spectrometry; ICP-OES, inductively coupled plasma optical emission spectroscopy; KD, knockdown.

ABSTRACT

The Sec61 protein translocon is a multimeric complex that transports proteins across lipid bilayers. We discovered that the Sec61 β subunit modulates cellular sensitivity to chemotherapeutic agents, particularly the platinum drugs. To investigate the mechanism, expression of Sec61 β was constitutively knocked down in 2008 ovarian cancer cells. Sec61 β knockdown (KD) resulted in 8-, 16.8- and 9-fold resistance to cisplatin (cDDP), carboplatin, and oxaliplatin, respectively. Sec61 β KD reduced the cellular accumulation of cDDP to 67% of the parental cells. Baseline copper (Cu), Cu uptake, and Cu cytotoxicity were also reduced. Given that Cu transporters and chaperones regulate Pt drug accumulation and efflux, their expression in 2008 Sec61 β KD cells was analyzed and ATP7A was found to be 2 to 3-fold over-expressed while there was no change in ATP7B, ATOX1, CTR1, or CTR2. Cells lacking ATP7A did not exhibit increased cDDP resistance upon knockdown of Sec61 β . Sec61 β KD cells also exhibited altered ATP7A cellular distribution. We conclude that Sec61 β modulates the cytotoxicity of many chemotherapeutic agents with the largest effect being on the platinum drugs. This is through its effect on the expression and distribution of ATP7A that has previously been shown to control Pt drug sequestration and cytotoxicity.

INTRODUCTION

While the ability of copper (Cu) to undergo reversible oxidation is essential for the function of Cu-requiring enzymes, this process produces reactive oxygen species that can cause severe cellular damage (Linder et al., 1996). Thus, cells have evolved a complex system of Cu transporters and chaperones that protect Cu(I) during its influx and distribution throughout the cytoplasm (Camakaris et al., 1999; Huffman et al., 2001; O'Halloran et al., 2000). ATP7A and ATP7B are P-type ATPases that sequester Cu into the trans-Golgi where it is loaded onto ceruloplasmin and other Cu-dependent enzymes (Dierick et al., 1997; Suzuki et al., 1999). Maintenance of the trans-Golgi compartments in which ATP7A and ATP7B reside may involve ADP-ribosylation factors (ARFs) and guanine nucleotide exchange factors (GEFs) (Holloway et al., 2007). Excess Cu causes ATP7A and ATP7B to relocate to either the plasma membrane or vesicular compartments and is thought to be necessary for the efflux or exocytosis of Cu (Camakaris et al., 1995; M. J. Petris et al., 1996; M.J. Petris et al., 1999; Roelofsen et al., 2000; Setty et al., 2008).

Resistance to the platinum (Pt) drugs can be the result of decreased drug uptake, changes in repair of DNA adducts, and alterations in apoptotic signaling pathways. We and others have shown that proteins involved in Cu homeostasis have been shown to control both the influx and efflux of the Pt-containing drugs (R. Safaei et al., 2005). CTR1 and CTR2 regulate uptake whereas ATP7A and ATP7B are involved in intracellular sequestration and drug export. Chaperones such as ATOX1 may transport the drugs between intracellular sites (B. G. Blair et al., 2009; A. Holzer et al., 2003; Roohangiz Safaei et al., 2007; Samimi, Katano, et al., 2003).

Cancer cell lines expressing high levels of ATP7A exhibit increased resistance to cDDP, carboplatin, and oxaliplatin (Samimi, Safaei, et al., 2004). Clinical data suggests that increased expression of ATP7A in tumors is associated with worse outcomes in ovarian and lung cancer patients treated with a Pt chemotherapeutic (Li et al., 2012; Samimi, Varki, et al., 2003). ATP7A contains several metal binding domains (MBD) that can bind cDDP, and one hypothesis is that ATP7A exports the Pt-drugs in a similar

manner to Cu. In addition to being associated with resistance to the Pt-drugs, ATP7A has also been shown to confer resistance to other chemotherapeutic agents. It is more difficult to explain how ATP7A might mediate resistance to less structurally related compounds and some have hypothesized that in addition to serving as a Cu export pump, ATP7A regulates an overall vesicle secretory process involved in the efflux of many compounds (Furukawa et al., 2008; Owatari et al., 2007).

The Sec61 translocon is an ER resident multimeric protein complex that allows the transport of proteins from one side of a membrane to another, or laterally into a lipid bilayer in the case of transmembrane proteins. It is composed of three subunits, α , β and γ , and structural data on the highly conserved bacterial secYEG suggests the α subunit forms the pore of the channel through which a polypeptide chain passes while the γ subunit stabilizes the structure (Van den Berg et al., 2004). The β subunit makes only peripheral contact with the complex and appears largely dispensable for the forward translocating function.

Less is known about retrotranslocation, but it has been reported that this involves the ER-associated degradation pathway (ERAD), and in some cases the β subunit has been shown to be important for this process (Liao et al., 2007; Scott et al., 2008). Sec61 β has also been associated with components of the exocyst complex and the reticulon family of proteins (J. H. Toikkanen et al., 2003; Zhao et al., 2009) whose main function is thought to be tethering of transport vesicles trafficked from the ER to the plasma membrane and shaping ER structure. Increased expression of Sec61 β has been reported in several types of cancer including ovary, kidney, prostate, and brain based on microarray data (Wu et al., 2009) although no one has examined how its expression changes during Pt drug therapy.

The importance of trafficking to the function of Cu export proteins led us to investigate the role of Sec61 β . We have made the novel observation that Sec61 β modulates sensitivity to the cytotoxic effect of the Pt chemotherapeutic agents. Knockdown of Sec61 β in ovarian cancer cells results in a large increase in resistance to cDDP, carboplatin, and oxaliplatin. Knockdown of Sec61 β also confers lower degrees of resistance to several other classes of clinical chemotherapeutics. Since resistance to the Pt-containing

drugs is linked to Cu homeostasis, we analyzed the effect of knocking down Sec61 β on expression of Cu transporters and chaperones and found a significant increase in the expression of ATP7A. Knockdown of Sec61 β in cells lacking ATP7A failed to alter drug sensitivity thus demonstrating a specific role for ATP7A. Further, the distribution of ATP7A is altered in Sec61 β KD cells. We conclude that Sec61 β modulates the cytotoxicity of many chemotherapeutic agents with the largest effect being on sensitivity to the Pt drugs, and this is mediated through an effect on the expression and subcellular localization of ATP7A.

MATERIALS AND METHODS

Drugs and reagents. Commercial formulations of cDDP, carboplatin and oxaliplatin were obtained from the Moores Cancer Center pharmacy. Doxorubicin, paclitaxel, etoposide and vincristine were gifts from the San Diego Veterans Affairs Infusion Center Pharmacy. The drugs were diluted to the desired concentrations in RPMI medium or alpha-MEM (Thermo Scientific; Logan, UT). The Detergent Compatible Protein kit was purchased from BioRad (Hercules, CA) and sulforhodamine B was obtained from Sigma-Aldrich (MP Biomedicals; Solon, OH); the sulforhodamine B dissolved to 0.4% (w/v) in 1% (v/v) acetic acid solution.

Cell types, culture, and molecular engineering. Human ovarian carcinoma 2008 (Disaia et al., 1972), IGROV-1 and A2780 cells were grown in RPMI medium supplemented with 10% fetal calf serum. Me32a and Me32a/MNK cell lines were grown in α MEM supplemented with 10% fetal calf serum. To constitutively suppress the expression of Sec61 β , three human ovarian carcinoma 2008 cells, IGROV-1 and A2780 cells and the human fibroblast cell line Me32a (Samimi, Katano, et al., 2003) were infected with a lentivirus expressing a short hairpin RNA targeting Sec61 β mRNA purchased from Sigma-Aldrich (St. Louis, MO). The sequence of the shRNA used for most knockdowns was ccggcagatattggtatgagtcttcctcgaggaagactcataaccaataactgtttttg. The shRNA sequences used for knockdowns targeted to other regions

of the Sec61 β gene were ccg ggattctacacagaagattcacctcgaggtgaatcttctgtgtagaatcttttttg and ccggcccaacat-ttcttgaccaactcgagtttg gtccaagaatgttggttttttg to create 2008 Sec61 β KD 279 and 927 sublines, respectively. Infected cells were selected in the appropriate medium containing 5 μ g/mL puromycin. Cell survival following exposure to increasing concentrations of drugs was assayed using the sulforhodamine B assay system (Monks et al., 1991). Four thousand cells were seeded into each well of a 96-well tissue culture plate. Cells were incubated overnight at 37 °C, 5% CO₂ and then exposed to varying drug concentrations in 200 μ L complete medium. Cells were allowed to grow for 4 days following the addition of drug after which the medium was removed and the protein was precipitated with 50% trichloroacetic acid and stained using 100 μ L of 0.4% sulforhodamine B in 1% acetic acid at room temperature for 15 min. After washing the absorbance of each well at 515 nm was recorded using a Versamax Tunable Microplate Reader (Molecular Devices; Sunnyvale, CA). Results are plotted as drug concentration versus log₁₀ cell survival. All experiments were repeated at least 3 times using 3 cultures for each drug concentration.

Western blotting. Whole-cell lysates were dissolved in lysis buffer [150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, and 10 mmol/L Tris (pH 7.4)] with protease inhibitor (Roche; Mannheim, Germany) and subjected to electrophoresis on 4% to 15% gels Tris-Glycine using 30-50 μ g of protein per lane. Protein levels were first determined by DC protein Assay (Bio-Rad; Hercules, CA). A Bio-Rad Trans-Blot system was used to transfer the proteins to Immobilon-P or Immobilon-P FL membranes (Millipore; Bedford, MA). Blots were incubated overnight at 4 °C in 5% dry nonfat milk in TBS-T [150 mmol/L NaCl, 300 mmol/L KCl, 10 mmol/L Tris (pH 7.4), 0.01% Tween 20] or Odyssey Blocking Buffer (Li-Cor; Lincoln, NE). Blots were incubated for 2 h at room temperature with anti-Sec61 β antibody at 1:1000 dilution (Upstate Cell Signaling Solutions; Lake Placid, NY), anti-ATP7A antibody at 1:500 dilution (Abcam; Cambridge, MA; or Neuromab; Davis, CA), anti-CTR2 antibody at 1:500 dilution (Custom antibody made by Biomatik against peptide SQQTIAETDGDSAGSD), anti-ATP7B at 1:1000 (Novus; Littleton, CO), anti-caveolin at 1:1000 (BD Biosciences; San Diego CA) or antibody to β -actin

(Santa Cruz; Santa Cruz, CA). A horseradish peroxidase–conjugated secondary antibody (GE Healthcare; United Kingdom) or fluorescently labeled secondary antibody (Li-Cor; Lincoln, NE) was dissolved in 5% milk in the TBS-T buffer or Odyssey Blocking Buffer and incubated with the blot for 1-2 h at room temperature. After three 10 min washes, blots were exposed to the Pierce enhanced chemiluminescence reagent (Thermo Scientific; Logan, UT) and detected on X-ray films (HyBlot CL, Denville Scientific, Inc.; Metuchen, NJ). Alternatively, blots probed with fluorescently labeled antibody were imaged using an Odyssey Infrared Imager (Li-Cor; Lincoln, NE).

Assessment of ATP7A protein stability. 2008 cells or 2008 Sec61 β KD cells were incubated with 30 μ g/mL cyclohexamide for 0, 12, 24, and 48 hours. Cells were then washed 3 times with PBS and lysates were harvested for Western blotting as described above. Bands were quantitated using the Odyssey Infrared Imager (Li-Cor; Lincoln, NE) and normalized to actin.

Lipid raft isolation. Fractions enriched in lipid rafts were prepared from the 2008 cells using a sodium carbonate-based detergent-free method as previously described (Ostrom et al., 2007). Briefly, 2008 cells were grown to 80-90% confluence, scraped into 2 mL 500 mM sodium carbonate and protease inhibitors. Homogenization was carried out using 10 strokes with a Dounce homogenizer, followed by four 20 second bursts with a probe sonicator. Homogenates were adjusted to 45% sucrose in 25 mM MES and 0.15 M NaCl. A 5-35% discontinuous sucrose gradient was layered over this in an ultracentrifuge tube, and spun at 39,000 rpm at 4 °C for 20 hours in a SW-40Ti rotor. From the top, 11 fractions were collected and analyzed by western blot as well as a total lysate aliquot.

Assessment of ATP7A cell surface expression. 2008 cells or 2008 Sec61 β KD cells were grown to ~80-90% confluence, then were incubated with 200 μ M CuSO₄. Cell surface protein was then biotinylated with the Pierce Cell Surface Protein Isolation Kit (Thermo Scientific; Logan, UT) per the manufacturers instructions. Samples were then analyzed by western blot as above. Antibodies to actin (Santa Cruz; Santa Cruz, CA) and Na,K-ATPase (Cell Signaling Technologies; Danvers, MA) were used as controls for quality of preparations.

qRT-PCR. Sec61 β mRNA levels were measured using a qRT-PCR method of detection of relative amounts of first-strand cDNA. cDNA was generated from mRNA isolated using TRIzol (Invitrogen; Carlsbad, CA). Purified mRNA was converted to cDNA using oligo(dT)₂₀ priming and the SuperScript III First-Strand kit (Invitrogen; Carlsbad, CA) qRT-PCR was performed on an MyIQ qPCR machine (Bio-Rad Laboratories; Hercules, CA). Reactions were prepared using iQ SYBR Green Supermix (Bio-Rad Laboratories; Hercules, CA) according to the manufacturer's recommendations. Samples were prepared in quadruplicate with 3 independent sample sets being analyzed. Analysis was done using the Bio-Rad iQ5 system software.

Measurement of Pt and Cu accumulation. Whole cell Pt and Cu content was measured as previously reported (Larson et al., 2009). Briefly cells were incubated in regular media or 30 μ M cDDP or 100 μ M CuSO₄ containing DMEM-RS media (Invitrogen; Hercules, CA) at 37°C for 60 min after which the drug-containing medium was removed and the cultures were washed 3 times with ice-cold HPLC grade PBS. To control for non-specific binding of drug to the wells and cell surface, time zero (Tz) samples, where drug-containing medium was aspirated within 15 s of the start of drug exposure, were also obtained, and this was subtracted from all measurements as previously described (Larson et al., 2009). Tz values were less than 2-3% of the level measured after 1 h of drug exposure.. Concentrated (70%) nitric acid was added to each well, and the plate was incubated overnight at room temperature to thoroughly dissolve all cellular debris. Following addition of an indium internal standard, platinum or Cu concentrations were measured using an Element 2 ICP-MS (Perkin-Elmer Life and Analytical Sciences; Waltham, MA) located at the Analytical Facility at Scripps Institute of Oceanography at the University of California (San Diego, CA). As a method of normalization, total sulfur was measured using an inductively coupled plasma optical emission spectroscopy (Perkin-Elmer Life and Analytical Sciences), also located at Scripps Institute of Oceanography at the University of California, San Diego. All data presented are the means of at least 3 independent experiments, each performed with 6 cultures per concentration tested.

For measurement of Pt in DNA, cells were lysed and DNA harvested using DNAzol (Invitrogen; Carlsbad, CA) according to the manufacturer's protocol. As a method of normalization, DNA was measured prior to addition of nitric acid using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). The DNA samples were then digested in nitric acid and prepared prior to measurement of Pt by ICP-MS as described above.

Deconvolution Microscopy. Cells were grown on 8-well microscope chamber slides (Millipore; Billerica, MA). Upon reaching ~60-80% confluence, the media was removed from each chamber. The chambers were then treated with either 300 μ l growth media containing either 200 μ M CuSO₄ or 100 μ M BCS for 1 h. Following 1h drug exposure, the media was removed and the slide was treated and then washed 3 times with PBS. Cells were fixed with 4% paraformaldehyde in PBS for 20 min followed by three 10 min PBS washes. Cells were then permeabilized with 0.3% Triton X in PBS followed by 50 m mol/L NH₄Cl in PBS for 10 min then two 10 min PBS washes. The slides were blocked for 1 h with 5% BSA in PBS and then treated with anti-ATP7A antibody (Neuromab; Davis, CA) at 1:250 dilution, syntaxin-6 1:500 (Cell Signaling Technology; Danvers, MA), and Hoechst 33342 at 1:20,000 followed by three 10 min PBS washes. The slides were then viewed using a Deltavision deconvolution microscope (Applied Percision, Inc. Issaquah, Washington).

Statistical analysis. All two-group comparisons utilized Student's *t*-test with the assumption of unequal variance. Data are presented as mean \pm SEM.

RESULTS

Creation of 2008 ovarian cancer Sec61 β knockdown cell line. Sec61 β expression was constitutively knocked down in the human cell ovarian cancer 2008 cell line by infecting with lentivirus expressing a shRNAi targeted to human Sec61 β . Following selection for successfully infected cells in puromycin, cultures grown from small infected populations of approximately 50-100 cells were screened by qRT-PCR and western blot analysis. As shown in Figure 1, in the culture selected for further analysis

there was a 96% reduction in mRNA expression (Figure 1A) and an 87% reduction in Sec61 β protein (Figure 1B).

Sec61 β controls sensitivity to cDDP, carboplatin and oxaliplatin. The parental 2008 ovarian cancer cells and the 2008 Sec61 β knockdown cells (2008 Sec61 β KD) were tested for sensitivity to the cytotoxic effect of the Pt-containing drugs using an SRB assay in which the cells were continuously exposed to increasing concentrations of cDDP, carboplatin, or oxaliplatin for 96 h (Monks et al., 1991). Figure 1C-E shows the concentration-survival curves for each of the cell lines for each of the Pt drugs. Knockdown of Sec61 β resulted in an 8-fold increase in resistance to cDDP as compared to the parental 2008 cells. The IC₅₀ (mean \pm SEM) was 2.6 ± 0.6 μ M for the parental 2008 cells and 21.6 ± 4.9 μ M for the 2008 Sec61 β KD cells ($p < 0.001$). Likewise, knockdown of Sec61 β increased resistance to carboplatin and oxaliplatin. The IC₅₀ for carboplatin increased 16.8-fold from 28.8 ± 0.6 in the parental 2008 cells to 462.1 ± 11.4 μ M in the knockdown cells ($p < 0.001$), and that for oxaliplatin increased resistance 9.6-fold from 0.33 ± 0.02 μ M to 2.49 ± 0.30 μ M ($p < 0.01$). These results indicate that Sec61 β modulates one or more defense mechanisms that offset the toxicity the Pt drugs.

To confirm this was not cell line- or shRNAi- specific, Sec61 β was constitutively knocked down in two other human ovarian cancer cell lines, IGROV-1 and A2780. Sec61 β mRNA was reduced by 98% and 82% and protein expression was reduced to 53% and 24% of that in the respective parental line in the IGROV-1 Sec61 β KD and A2780 Sec61 β KD cells, respectively (Supplemental Table 1 and Supplemental Figure S1). Both lines demonstrated an increased level of cDDP resistance. The IC₅₀ (mean \pm SEM) was 0.48 ± 0.06 μ M for the parental IGROV-1 cells and 2.54 ± 0.01 μ M for the IGROV-1 Sec61 β KD cells reflecting a 5-fold increase ($p < 0.001$) in resistance. The IC₅₀ (mean \pm SEM) was 3.82 ± 0.27 μ M for the parental A2780 cells and 6.42 ± 0.27 μ M for the A2780 Sec61 β KD cells reflecting a significant 1.7-fold increase ($p < 0.05$) in resistance. Additional 2008 Sec61 β KD lines were also generated using shRNA lentiviral vectors targeted to other segments of the Sec61 β gene to generate two additional 2008 Sec61 β KD lines, 2008 Sec61 β KD 279 and 2008 Sec61 β KD 927. Knockdown in these

lines was confirmed by qRT-PCR. They were found to be 9.3-fold and 14.0-fold resistant to cDDP, respectively (Table S1). Thus, knockdown of Sec61 β mRNA consistently produced resistance to cDDP in multiple human ovarian cancer cell lines, and targeting different parts of the mRNA reproduced the same phenotype in the 2008 cells.

Sec61 β knockdown impairs cDDP accumulation. To define the mechanism by which Sec61 β influences sensitivity to cDDP in more detail, the whole cell content of Pt in the parental 2008 and 2008 Sec61 β KD cells was measured following a 1 h exposure to 30 μ M cDDP by ICP-MS. Whereas the parental 2008 cells accumulated 2.18 ± 0.01 ng Pt/ μ g S the knockdown cells accumulated 1.46 ± 0.03 ng Pt/ μ g S, a 33% reduction in the total accumulation of Pt drug (Figure 2A). Thus, at least part of the observed reduction in drug sensitivity can be accounted for by a decrease in drug accumulation.

Formation of DNA-Pt adducts is believed to be the primary mechanism by which the Pt drugs cause cell cytotoxicity. Pt was measured in DNA isolated from cells exposed for 1 h to 30 μ M cDDP (Figure 2B). The 2008 Sec61 β KD cells contained 14% less Pt in their DNA, 8.65 ± 0.64 pg Pt/ μ g, than the parental cells, 10.05 ± 0.65 pg Pt/ μ g DNA, although this difference was not statistically significant ($p = 0.22$).

Sec61 β knockdown increases the level of ATP7A. Given the observation that knockdown of Sec61 β rendered cells resistant to cDDP, and that sensitivity to cDDP is mediated in part by Cu transporters and chaperones, the relative expression of Cu transporters and chaperones previously shown to be capable of modulating cellular pharmacokinetics of cDDP was quantified by qRT-PCR and western blot analysis (Figure 3). The mRNA level of ATP7A was 2.1 ± 0.4 - fold higher in the 2008 Sec61 β KD cells, but there were no significant changes in the expression of mRNA for ATOX1, ATP7B, CTR1, or CTR2 (Figure 3A). Consistent with the change in mRNA level, the level of ATP7A protein was 2.5 ± 0.3 -fold higher in the Sec61 β knockdown cells when assessed by western blot (Figure 3B). No significant differences were found in the levels of ATOX1, ATP7B or CTR2 on western blot analysis; CTR1 was not tested due to lack of a reliable antibody capable of detecting the endogenous protein in these cells. The

ATP7A protein level was also increased in the other cell lines in which Sec61 β expression was knocked down. It was increased by 3.8 ± 1.8 -fold in the IGROV-1 Sec61 β KD cells and 2.9 ± 1.7 -fold in the A2780 Sec61 β KD cells compared to their respective parental cells (Figure S1).

To determine whether the ability of Sec61 β knockdown to increase ATP7A protein expression was due only to a change in ATP7A mRNA level, the stability of ATP7A protein was assessed by blocking new protein synthesis in the 2008 and 2008 Sec61 β KD cells with cycloheximide and monitoring the disappearance of ATP7A by serial western blot analysis. ATP7A was found to be very stable with a half-life >48 h similar to what has been reported by others (Holloway et al., 2007; Pase et al., 2004). In comparing the rate of ATP7A degradation between the parental and 2008 Sec61 β KD cells, no significant difference was detected (Figure 3C and D). With the caution that a long exposure to cycloheximide can affect a variety of cellular processes, this result suggests that the increase in ATP7A protein resulting from Sec61 β knockdown is due to an increase in mRNA level and not due to a change protein stability.

Sec61 β knockdown perturbs Cu homeostasis. ATP7A is responsible for pumping intracellular Cu into the secretory compartment and hence out of the cell, and its over-expression is associated with reduced accumulation of Cu and resistance to its cytotoxic effect. To determine whether the change in ATP7A protein expression was sufficient to perturb Cu homeostasis, whole cell Cu content was measured by ICP-MS in the parental and 2008 Sec61 β KD cells. As shown in Figure 4A, the basal Cu level in the 2008 Sec61 β KD cells was 40% lower than in the parental cells. The 2008 Sec61 β KD cells contained 11.8 ± 1.3 ng Cu/ μ g S whereas the parental cells contained 19.1 ± 2.4 ng Cu/ μ g S, a statistically significant difference ($p < 0.05$). To measure the effect on Cu accumulation, the cells were exposed to 100 μ M CuSO₄ for 1 h, washed and whole cell Cu content measured. Net uptake following Cu exposure was 31.6 ± 1.8 ng Cu/ μ g S versus 18.3 ± 1.3 ng Cu/ μ g S in the 2008 and 2008 Sec61 β KD cells respectively ($p < 0.01$). Thus, knock down of Sec61 β reduced both basal Cu content and the rate of Cu accumulation consistent with enhanced export of Cu mediated by the elevated level of ATP7A.

The parental and Sec61 β knock down cells were also tested for their sensitivity to Cu. The concentration-survival curves shown in Figure 4B indicate that the knockdown cells were 4.6-fold resistant to CuSO₄ with the parental cells exhibiting an IC₅₀ of 36.1 \pm 0.1 μ M and the knockdown cells 166.0 \pm 0.1 μ M ($p < 0.01$). Thus, the 2.5-fold increase in ATP7A protein produced by Sec61 β knock down was sufficient to significantly reduce initial Cu accumulation and render cells quite resistant to the growth inhibitory effect of Cu, indicating that the excess ATP7A was fully functional with respect to Cu homeostasis.

Sec61 β controls sensitivity to other chemotherapeutics. Over-expression of ATP7A has previously been reported to render cells resistant to several other classes of chemotherapeutic drugs in addition to the Pt-containing agents (Furukawa et al., 2008; Owatari et al., 2007). To determine whether loss of Sec61 β modulated sensitivity to other classes of drugs in human ovarian cancer the parental and Sec61 β knockdown 2008 cells were exposed to increasing concentrations of etoposide, vincristine, doxorubicin, and paclitaxel, each of which belongs to a different mechanistic class. As shown in Table 1, loss of Sec61 β function reduced sensitivity to all of these drugs but the magnitude of resistance was substantially less than for the Pt drugs. Since each of these drugs has a different mechanism by which it injures cells, this result indicates that Sec61 β participates in some mechanism of cellular resistance common to all of these agents.

Cu and cDDP do not alter expression levels of ATP7A. We and others have shown that expression of some Cu transporters such as CTR1 vary in response to changes in the availability of Cu or Pt drug exposure. This presumably serves to buffer the cell from high or low levels of environmental Cu (B. Blair et al., 2010; Y. Guo et al., 2004; Larson et al., 2009). To determine whether Cu or cDDP acutely influence the level of expression of ATP7A, the parental 2008 and Sec61 β KD cells were exposed to 200 μ M CuSO₄, 100 μ M of the Cu chelator BCS, or 30 μ M cDDP for one 1 h and ATP7A levels were assessed by western blot. As shown in Figure 5, the increase in ATP7A protein expression was maintained in all of the three cell lines comparing the parental 2008 versus the 2008 Sec61 β KD cells.

Under all conditions tested, the 2008 Sec61 β KD cells maintained a higher expression of ATP7A over the similarly treated parental 2008 cells. While quantification of protein band density suggested a possible higher expression of ATP7A in Sec61 β KD cells following CuSO₄ treatment of 5.4 ± 3 -fold over the parental 2008s, this was not statistically greater than the 2 to 2.4 fold increase seen in with the other three conditions. Thus, it appears that the effect on ATP7A expression mediated by Sec61 β KD is not sensitive to acute changes in extracellular Cu levels although changes over extended periods of exposure cannot be ruled out. This is in contrast to known effects on other Cu transporters such as CTR1 and CTR2 whose expression is rapidly altered when exposed to excess Cu or Cu chelators which has been reported to occur over a matter of minutes (B. Blair et al., 2010; A. K. Holzer et al., 2004).

Resistance to Pt drugs mediated by Sec61 β KD requires ATP7A expression. If the change in sensitivity mediated by Sec61 β KD requires ATP7A expression, then cells lacking ATP7A should have a similar sensitivity to cDDP regardless of Sec61 β expression. The Me32a cell line was derived from fibroblasts from a Menkes disease patient in which a mutation has inactivated ATP7A by creation of a premature stop codon (La Fontaine et al., 1998). Sec61 β was knocked down in the Me32a cell line by lentiviral expression of an shRNA directed against Sec61 β in a manner similar to that previously described above, thus creating Me32a Sec61 β KD cells. Knockdown of Sec61 β in these cells was confirmed by qRT-PCR and western blot (Figure 6A and B). Although no ATP7A protein was detectable in the Me32a or Me32a Sec61 β KD cells by western blot as expected, a 2.3 ± 0.4 -fold increase in ATP7A mRNA was still detected by qRT-PCR suggesting the transcriptional effects of Sec61 β KD were still operative (Figure 6B). Knockdown of Sec61 β in the Me32a cell line did not result in any appreciable change in cDDP sensitivity (Figure 6C). This result provides evidence that a functional ATP7A is required for knock down of Sec61 β to produce cDDP resistance.

Knockdown of Sec61 β disrupts the normal cellular localization of ATP7A. ATP7A resides in the trans-Golgi in the absence of extracellular Cu. Previously, we and others have shown that ATP7A redistributes from this perinuclear localization to other locations near or at the cell surface upon exposure

to excess levels of Cu (M. J. Petris et al., 1996; Samimi, Katano, et al., 2004), a process that may direct Cu to export pathways. Maintenance of the compartment responsible for ATP7A localization is in part due to activity of ARFs and GEFs (Holloway et al., 2007). In contrast, cDDP does not cause subcellular redistribution in response to Cu, but presumably a higher level of ATP7A in the cell may still help to sequester the drug into cellular vesicles thereby limiting its cytotoxicity. The distribution of ATP7A was examined by deconvolution microscopy in parental and 2008 Sec61 β KD cells. In the parental cells ATP7A was concentrated in perinuclear structures and co-localized with the trans-Golgi marker syntaxin-6 when grown in medium without added Cu (Figure 7). Upon exposure to 200 μ M CuSO₄, ATP7A redistributed throughout the cell including areas close to the plasma membrane similar to what has been reported previously. ATP7A failed to significantly co-localize with the other endosomal markers EEA1, rab11, or rab4 in either the parental or 2008 Sec61 β KD cells (Supplemental Figures S2-S7). Knockdown of Sec61 β altered the localization of ATP7A; it was no longer concentrated in perinuclear structures and failed to co-localize with syntaxin-6 to the same degree as in the parental cells (Figure 7). Interestingly, syntaxin-6 staining was also more diffuse in the 2008 Sec61 β KD cells raising the question of whether the altered distribution of ATP7A was due to a disturbance in the structure of the trans-Golgi. Knockdown of Sec61 β did not alter the subcellular distribution of EEA1, rab11, or rab4. Exposure of 2008 Sec61 β KD cells to 200 μ M CuSO₄ did not appreciably alter the distribution of ATP7A which was already diffusely distributed throughout the cell as detected by microscopy. Immunoprecipitation of Sec61 β failed to precipitate ATP7A and vice versa suggesting that these two proteins were not stably binding to each other (data not shown).

Given that a proportion of ATP7A has been shown to reside in membrane lipid rafts (Ashino et al., 2010), we sought to determine whether Sec61 β was also present within these same fractions. Parental 2008 cells were lysed and fractions containing lipid rafts were isolated by detergent-free sucrose density centrifugation (Ashino et al., 2010; Ostrom et al., 2007). Similar to what has been reported previously, ATP7A was found in fractions containing the lipid raft marker caveolin (Figure 8). Interestingly, Sec61 β

was also preferentially enriched in the same raft fraction, consistent with the possibility that it plays a role in the partitioning or trafficking of ATP7A in lipid rafts.

Although ATP7A was no longer localized to the trans-Golgi in cells in which Sec61 β was knocked down, this did not preclude the possibility that ATP7A could still traffic to plasma membrane sites in response to Cu. To determine whether Sec61 β was required for the trafficking of ATP7A to the plasma membrane, the amount of ATP7A on the cell surface susceptible to biotinylation was quantified before and after exposure to 200 μ M CuSO₄. As shown in Figure 9, plasma membrane ATP7A was approximately 1.8-fold higher in untreated 2008 Sec61 β KD cells as compared to the parental 2008 cells. Cu exposure increased plasma membrane ATP7A approximately 2-fold in the parental cells, an observation consistent with a previous report (Nyasae et al., 2007). It also increased cell surface ATP7A in the 2008 Sec61 β KD cells by a similar proportion indicating that Sec61 β is not required for the plasma membrane insertion step. These results demonstrate that Sec61 β plays a pivotal role in controlling the subcellular distribution of ATP7A as well as the level of its expression, and that both proteins are located in membrane fraction containing lipid rafts. Loss of Sec61 β does not disable the ability of ATP7A to sense and traffic to the plasma membrane in response to Cu.

DISCUSSION

We have made the novel observation that Sec61 β produces large changes in sensitivity to the cytotoxic effect of the Pt-drugs. In pursuit of the mechanism, it was discovered that knockdown of Sec61 β resulted in increased expression of ATP7A at both the RNA and protein level and an altered subcellular distribution. Resistance to the Pt-drugs induced by Sec61 β knockdown is mediated by ATP7A as evidenced by the fact that knockdown of Sec61 β in a cell in which ATP7A was non-functional failed to induce resistance. Additional evidence consistent with a central role for ATP7A includes

reduced basal Cu content, impaired Cu accumulation, concomitant resistance to Cu cytotoxicity, reduced cDDP uptake and a trend toward reduced adduct formation.

ATP7A has previously been shown to mediate resistance to Cu, the Pt-containing drugs cisplatin, carboplatin, and oxaliplatin, as well as other chemotherapeutics (Furukawa et al., 2008; Samimi, Katano, et al., 2004; Samimi, Safaei, et al., 2004). In the case of Cu, ATP7A mediates resistance by sequestering the metal into the vesicles of the secretory system and thus enhancing export. Cu is known to trigger the relocalization of ATP7A-expressing vesicles from the Golgi to a region just under or at the plasma membrane whence it is presumed that direct plasma membrane export or exocytosis of Cu occurs (Nyasae et al., 2007; M. J. Petris et al., 1996). In the case of cDDP, which unlike Cu does not trigger the relocalization of ATP7A-expressing vesicles, the mechanism by which ATP7A mediates resistance is less clear. In some cases ATP7A appears to work primarily by detoxifying cDDP through intracellular sequestration into ATP7A-expressing vesicles and this may increase rates of drug exocytosis (Katano et al., 2002; Samimi, Katano, et al., 2004). Whether these vesicles are distinct from those associated with the trans-Golgi or some other specific intracellular compartment is unknown.

The number of identified functions of the Sec61 translocon, of which Sec61 β is a part, is growing. It seems unlikely that forward translocation is the function of Sec61 that is related to chemotherapy drug resistance observed in this study as the Sec61 β subunit has been found to be largely unnecessary for forward translocation function (Gorlich et al., 1993; J. Toikkanen et al., 1996). However, the channel also functions in the reverse direction for the retrotranslocation and degradation of misfolded ER-resident or transmembrane proteins such as the cystic fibrosis transmembrane conductance regulator (CFTR) (Bebok et al., 1998). More recent work has shown that retrotranslocation is utilized not only for degradation, but also to manage cellular signaling pathways (Liao et al., 2007). Interestingly, as opposed to forward translocation, retrotranslocation is more dependent on the function of Sec61 β suggesting this subunit plays a more important role during the reverse process. Sec61 β has been shown to physically interact with some of these retrotranslocated proteins such as the EGF receptor and Gurken (Kelkar et al.,

2009; Liao et al., 2007). In the case of ATP7A we were unable to demonstrate such an interaction through co-immunoprecipitation experiments, and knockdown of Sec61 β did not change the stability of ATP7A arguing against a direct role for Sec61 β in its degradation. Although we show here that the ability of Sec61 β knockdown to mediate resistance to the Pt drugs is dependent on ATP7A, it is possible that effects on other proteins which rely on the Sec61 translocon for retrotranslocation or degradation also contribute to the resistant phenotype.

The Sec61 β subunit also has functions not necessarily tied to protein translocation. Sec61 β appears to be highly conserved since human Sec61 β can complement loss of the homologous yeast protein Sbh1p (Leroux et al., 2008). Sbh1p associates with components of the exocyst complex and the reticulon family of proteins (J. H. Toikkanen et al., 2003; Zhao et al., 2009) which are involved in directing vesicles from the Golgi to specific locations at the plasma membrane and tethering them there prior to vesicle fusion. Exocyst proteins have also been linked to control of cell polarity and cytoskeleton (Braun et al., 2010; W. Guo et al., 2004; He et al., 2009; Lipschutz et al., 2003). In yeast, the Sec61 β homologue Sbh1p was shown to physically interact with exocyst complex proteins such as Sec15p and Sec8p, and over-expression of Sbh1p suppressed growth defects in various exocyst mutants suggesting it plays a central role in exocyst function (J. H. Toikkanen et al., 2003).

The finding that knockdown of Sec61 β led to apparent dissolution of the trans-Golgi compartment associated with ATP7A, and the fact that ATP7A still retained its ability to traffic to the plasma membrane in response to Cu, is a very similar to the phenotype reported by Holloway et al. (Holloway et al., 2007). In their study of yeast, knockdown of tethering factor p115 led to dissolution of trans-Golgi structures associated with ATP7A but this did not interfere with the ability of ATP7A to traffic in response to elevated Cu. In the same study, they showed that expression of a dominant negative GEF of GBF1, which can interact with ARF1, also led to dissolution of the trans-Golgi and ATP7A-containing compartments although Cu responsive trafficking was lost. Strikingly, the Sec61 β yeast homologues sbh1p and 2p were shown in a different study to function as GEFs consistent with the fact that they

contain homologous sec7 domains required for GEF function (Helmers et al., 2003). Our data suggests the possibility that Sec61 β may function as a GEF, and could be modulating the ATP7A trans-Golgi compartment in a manner similar to the yeast GEF GBF1, possibly in concert with the ARF family of proteins. If this is the case, it may be that compartments “loaded” with Pt-drug by ATP7A are more efficient at sequestration or export when more diffusely distributed in Sec61 β KD cells.

ATP7A function is largely regulated by post-translational control of trafficking. When Cu is abundant it is transported in vesicles to areas at or near the plasma membrane. ATP7A-containing vesicles can also re-localize to the leading edge in vascular smooth muscle cells upon stimulation of migration with PDGF or following wounding of cell culture (Ashino et al., 2010). Elements of the exocyst complex are likely involved in the transport of ATP7A-containing vesicles and knockdown of Sec61 β probably interferes with this process either by impairing the exocyst or by altering trans-Golgi structure. Whether Sec61 β controls the loading of ATP7A into vesicles bound for export or the trafficking of vesicles already loaded by ATP7A with Pt drugs is still unknown. It is notable that knockdown of Sec61 β increases ATP7A expression in addition to altering its cellular distribution. While the increase in ATP7A expression could represent a direct effect of Sec61 β knockdown and resultant overwhelming of normal trafficking mechanisms, the higher expression could also reflect a cellular response to disrupted ATP7A localization although our data in the Me32a Sec61 β KD cells would not support this. As no clear physical interaction could be demonstrated between ATP7A and Sec61 β by either immunoprecipitation or colocalization by microscopy (Supplemental Figures S8 and data not shown), it appears unlikely that Sec61 β controls ATP7A localization through a direct association, but rather through effects on other aspects of the cellular trafficking machinery. The enrichment of both proteins in membrane fractions containing lipid rafts is supportive of this hypothesis. It is noteworthy that Sec61 β knockdown did not alter the expression of other Cu homeostasis proteins and produced no large effects on cell growth or morphology.

Knockdown of Sec61 β resulted in greater resistance to the Pt-containing drugs than to Cu. Prior studies have repeatedly shown that relatively small increments of resistance to Cu are accompanied by substantially larger increments of resistance to the Pt-containing drugs (R. Safaei et al., 2005). Unlike Cu, cDDP does not trigger the relocalization of ATP7A from the Golgi apparatus (Samimi, Katano, et al., 2004; Samimi, Varki, et al., 2003). Nevertheless, expression of wild type ATP7A in fibroblasts that contain no endogenous ATP7A, or over-expression in ovarian cancer cells, results in resistance to cDDP (Komatsu et al., 2000; Owatari et al., 2007; Samimi, Katano, et al., 2004; Samimi, Varki, et al., 2003). The finding of cross resistance between Cu and cDDP led to recognition that the resistance was tied to drug accumulation as opposed to downstream effects on apoptosis. The resistance to cDDP produced by knockdown of Sec61 β was associated with increased levels of cell surface ATP7A, a ~30% reduction in cDDP accumulation and a trend toward reduced DNA adduct formation, all consistent with enhanced ATP7A-mediated export. It has previously been noted that very small changes in the extent of adduct formation are associated with relatively large changes in Pt drug sensitivity (Johnson et al., 1997).

How Sec61 β affects resistance to the other chemotherapeutics tested is still unclear. The results obtained in the 2008 cells confirm earlier observations that increased expression of ATP7A, including in cells that over-express ATP7A following transfection, is associated with resistance to various other classes of chemotherapeutic agents (Furukawa et al., 2008; Owatari et al., 2007). While, like ATP7B (R. Safaei et al., 2008), ATP7A may directly transport cDDP, it seems unlikely that it transports all of the drugs for which resistance has been demonstrated and it is more likely that Sec61 β either controls other types of transporters, alters vesicle pH, or that Sec61 β or ATP7A alter the activity of the vesicle secretory pathway. These hypotheses remain to be tested. It is notable that ATP7A is involved in cellular migration (Ashino et al., 2010; Furukawa et al., 2008), as migration also involves trafficking and cytoskeletal modifications which likely depend on processes analogous to vesicle trafficking and exocytosis.

In conclusion, we have shown that Sec61 β has large effects on cellular sensitivity to the Pt drugs and a more modest effect on other classes of chemotherapeutics. In the case of the Pt drugs resistance is

ATP7A-dependent and at least partially due to a change in Pt drug accumulation. Sec61 β is required for the trans-Golgi localization of ATP7A, but not for the ability of ATP7A to respond to Cu. The mechanism by which Sec61 β alters ATP7A levels is a subject for future study.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Abada, P.B. Larson, C.A. and Howell S.B.

Conducted experiments: Abada, P.B. Larson, C.A., Manorek G., Adams, P.

Contributed new reagents or analytic tools: Abada, P.B.

Performed data analysis: Abada, P.B. and Howell S.B.

Wrote or contributed to the writing of the manuscript: Abada, P.B. and Howell S.B.

REFERENCES

Ashino, T., Sudhahar, V., Urao, N., Oshikawa, J., Chen, G. F., Wang, H., Huo, Y., Finney, L., Vogt, S., McKinney, R. D., Maryon, E. B., Kaplan, J. H., Ushio-Fukai, M., & Fukai, T. (2010). Unexpected role of the copper transporter ATP7A in PDGF-induced vascular smooth muscle cell migration. *Circ Res*, 107(6), 787-799.

Bebok, Z., Mazzochi, C., King, S. A., Hong, J. S., & Sorscher, E. J. (1998). The mechanism underlying cystic fibrosis transmembrane conductance regulator transport from the endoplasmic reticulum to the proteasome includes Sec61beta and a cytosolic, deglycosylated intermediary. *J Biol Chem*, 273(45), 29873-29878.

Blair, B., Larson, C., Adams, P., Abada, P., Safaei, R., & Howell, S. (2010). Regulation of CTR2 expression by copper and cisplatin in human ovarian carcinoma cells. *Mol Pharmacol*, 77, 912 - 921.

Blair, B. G., Larson, C. A., Safaei, R., & Howell, S. B. (2009). Copper transporter 2 regulates the cellular accumulation and cytotoxicity of cisplatin and carboplatin. *Clin Cancer Res*, 15(13), 4312-4321.

Braun, V., & Brumell, J. H. (2010). Bacterial invasion: entry through the exocyst door. *Curr Biol*, 20(16), R677-679

Camakaris, J., Petris, M. J., Bailey, L., Shen, P., Lockhart, P., Glover, T. W., Barcroft, C., Patton, J., & Mercer, J. F. (1995). Gene amplification of the Menkes (MNK; ATP7A) P-type ATPase gene of CHO cells is associated with copper resistance and enhanced copper efflux. *Hum Mol Genet*, 4(11), 2117-2123.

Camakaris, J., Voskoboinik, I., & Mercer, J. F. (1999). Molecular mechanisms of copper homeostasis. *Biochem Biophys Res Commun.*, 261(2), 225-232.

Dierick, H. A., Adam, A. N., Escara-Wilke, J. F., & Glover, T. W. (1997). Immunocytochemical localization of the Menkes copper transport protein (ATP7A) to the trans-Golgi network. *Hum Mol Genet*, 6(3), 409-416.

Disaia, P. J., Sinkovics, J. G., Rutledge, F. N., & Smith, J. P. (1972). Cell-mediated immunity to human malignant cells. *Am J Obstet Gynecol*, 114, 979-989.

Furukawa, T., Komatsu, M., Ikeda, R., Tsujikawa, K., & Akiyama, S. (2008). Copper transport systems are involved in multidrug resistance and drug transport. *Curr Med Chem*, 15(30), 3268-3278.

Gorlich, D., & Rapoport, T. A. (1993). Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane. *Cell*, 75(4), 615-630.

Guo, W., & Novick, P. (2004). The exocyst meets the translocon: a regulatory circuit for secretion and protein synthesis? *Trends Cell Biol*, 14(2), 61-63.

Guo, Y., Smith, K., Lee, J., Thiele, D. J., & Petris, M. J. (2004). Identification of methionine-rich clusters that regulate copper-stimulated endocytosis of the human Ctr1 copper transporter. *J Biol Chem*, 279, 17428-17433.

He, B., & Guo, W. (2009). The exocyst complex in polarized exocytosis. *Curr Opin Cell Biol*, 21(4), 537-542.

Helmers, J., Schmidt, D., Glavy, J. S., Blobel, G., & Schwartz, T. (2003). The beta-subunit of the protein-conducting channel of the endoplasmic reticulum functions as the guanine nucleotide exchange factor for the beta-subunit of the signal recognition particle receptor. *J Biol Chem*, 278(26), 23686-23690.

Holloway, Z. G., Grabski, R., Szul, T., Styers, M. L., Coventry, J. A., Monaco, A. P., & Sztul, E. (2007). Activation of ADP-ribosylation factor regulates biogenesis of the ATP7A-containing trans-Golgi network compartment and its Cu-induced trafficking. *Am J Physiol Cell Physiol*, 293(6), C1753-1767.

Holzer, A., Samimi, G., Katano, K., Naedermann, W., & Howell, S. B. (2003). The role of human copper transporter hCTR1 in cisplatin uptake in human ovarian carcinoma cells. *Proc Amer Assoc Cancer Res.*, 44, 923.

Holzer, A. K., Katano, K., Klomp, L. W., & Howell, S. B. (2004). Cisplatin rapidly down-regulates its own influx transporter hCTR1 in cultured human ovarian carcinoma cells. *Clin Cancer Res*, 10(19), 6744-6749.

Huffman, D. L., & O'Halloran, T. V. (2001). Function, structure, and mechanism of intracellular copper trafficking proteins. *Annu Rev Biochem*, 70, 677-701.

Johnson, S. W., Laub, P. B., Beesley, J. S., Ozols, R. F., & Hamilton, T. C. (1997). Increased platinum-DNA damage tolerance is associated with cisplatin resistance and cross-resistance to various chemotherapeutic agents in unrelated human ovarian cancer cell lines. *Cancer Res.*, 57, 850-856.

Katano, K., Kondo, A., Safaei, R., Holzer, A., Samimi, G., Mishima, M., Kuo, Y. M., Rochdi, M., & Howell, S. B. (2002). Acquisition of resistance to cisplatin is accompanied by changes in the cellular pharmacology of copper. *Cancer Res*, 62(22), 6559-6565.

Kelkar, A., & Dobberstein, B. (2009). Sec61beta, a subunit of the Sec61 protein translocation channel at the endoplasmic reticulum, is involved in the transport of Gurken to the plasma membrane. *BMC Cell Biol*, 10, 11.

Komatsu, M., Sumizawa, T., Mutoh, M., Chen, Z.-S., Terada, K., Furukawa, T., Yang, X.-L., Gao, H., Miura, N., Sugiyama, T., & Akiyama, S. (2000). Copper-transporting P-type adenosine triphosphatase (ATP7B) is associated with cisplatin resistance. *Cancer Res*, 60(March 1, 2000), 1312-1316.

La Fontaine, S. L., Firth, S. D., Camakaris, J., Englezou, A., Theophilos, M. B., Petris, M. J., Howie, M., Lockhart, P. J., Greenough, M., Brooks, H., Reddel, R. R., & Mercer, J. F. (1998). Correction of the copper transport defect of Menkes patient fibroblasts by expression of the Menkes and Wilson ATPases. *J Biol Chem*, 273(47), 31375-31380.

Larson, C. A., Blair, B. G., Safaei, R., & Howell, S. B. (2009). The role of the mammalian copper transporter 1 in the cellular accumulation of platinum-based drugs. *Mol Pharmacol*, 75(2), 324-330.

Leroux, A., & Rokeach, L. A. (2008). Inter-species complementation of the translocon beta subunit requires only its transmembrane domain. *PLoS One*, 3(12), e3880.

Li, Z. H., Qiu, M. Z., Zeng, Z. L., Luo, H. Y., Wu, W. J., Wang, F., Wang, Z. Q., Zhang, D. S., Li, Y. H., & Xu, R. H. (2012). Copper-transporting P-type adenosine triphosphatase (ATP7A) is associated with platinum-resistance in non-small cell lung cancer (NSCLC). *J Transl Med*, 10, 21.

Liao, H. J., & Carpenter, G. (2007). Role of the Sec61 translocon in EGF receptor trafficking to the nucleus and gene expression. *Mol Biol Cell*, 18(3), 1064-1072.

Linder, M. C., & Hazegh-Azam, M. (1996). Copper biochemistry and molecular biology. *Am J Clin Nutr*, 63(5), 797S-811S.

Lipschutz, J. H., Lingappa, V. R., & Mostov, K. E. (2003). The exocyst affects protein synthesis by acting on the translocation machinery of the endoplasmic reticulum. *J Biol Chem*, 278(23), 20954-20960.

Monks, A., Scudiero, D., Skehan, P., Shoemaker, R., Paull, K., Vistica, D., Hose, C., Langley, J., Cronise, P., Vaigro-Wolff, A., Gray-Goodrich, M., Campbell, H., Mayo, J., & Boyd, M. (1991). Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Natl Cancer Inst*, 83, 757-765.

Nyasae, L., Bustos, R., Braiterman, L., Eipper, B., & Hubbard, A. (2007). Dynamics of endogenous ATP7A (Menkes protein) in intestinal epithelial cells: copper-dependent redistribution between two intracellular sites. *Am J Physiol Gastrointest Liver Physiol*, 292(4), G1181-1194.

O'Halloran, T. V., & Culotta, V. C. (2000). Metallochaperones, an intracellular shuttle service for metal ions. *J Biol Chem*, 275(33), 25057-25060.

Ostrom, R. S., & Liu, X. (2007). Detergent and detergent-free methods to define lipid rafts and caveolae. [Review]. *Methods Mol Biol*, 400, 459-468.

Owatari, S., Akune, S., Komatsu, M., Ikeda, R., Firth, S. D., Che, X. F., Yamamoto, M., Tsujikawa, K., Kitazono, M., Ishizawa, T., Takeuchi, T., Aikou, T., Mercer, J. F., Akiyama, S., & Furukawa, T. (2007). Copper-Transporting P-Type ATPase, ATP7A, Confers Multidrug Resistance and Its Expression Is Related to Resistance to SN-38 in Clinical Colon Cancer. *Cancer Res*, 67(10), 4860-4868.

Pase, L., Voskoboinik, I., Greenough, M., & Camakaris, J. (2004). Copper stimulates trafficking of a distinct pool of the Menkes copper ATPase (ATP7A) to the plasma membrane and diverts it into a rapid recycling pool. *Biochem J*, 378(Pt 3), 1031-1037.

Petris, M. J., Mercer, J. F., Culvenor, J. G., Lockhart, P., Gleeson, P. A., & Camakaris, J. (1996). Ligand-regulated transport of the Menkes copper P-type ATPase efflux pump from the Golgi apparatus to the plasma membrane: a novel mechanism of regulated trafficking. *Embo J*, 15(22), 6084-6095.

Petris, M. J., & Mercer, J. F. B. (1999). The Menkes protein (ATP7A;MNK) cycles via the plasma membrane both in basal and elevated extracellular copper using a C-terminal di-leucine endocytic signal. *Hum Mol Genet*, 8(11), 2107-2115.

Roelofsen, H., Wolters, H., Van Luyn, M. J., Miura, N., Kuipers, F., & Vonk, R. J. (2000). Copper-induced apical trafficking of ATP7B in polarized hepatoma cells provides a mechanism for biliary copper excretion. *Gastroenterology*, 119(3), 782-793.

Safaei, R., & Howell, S. B. (2005). Copper transporters regulate the cellular pharmacology and sensitivity to Pt drugs. *Crit Rev Oncol Hematol*, 53(1), 13-23.

Safaei, R., Larson, B. J., Otani, S., Rasmussen, M. L., & Howell, S. B. (2008). Transport of cisplatin by the copper efflux transporter ATP7B. *Mol Pharmacol*, 73, 461-468.

Safaei, R., Rasmussen, M. L., Francisco, K. S., & Howell, S. B. (2007). The copper chaperone Atox1 is involved in the intracellular sequestration of cisplatin. Paper presented at the Proc Amer Assoc Cancer Res.

Samimi, G., Katano, K., Holzer, A., Safaei, R., Petris, M. J., & Howell, S. B. (2003). The copper export transporters ATP7A and ATP7B modulate the cellular pharmacology of cisplatin and carboplatin. *Proc Amer Assoc Cancer Res*, 44, 806-807.

Samimi, G., Katano, K., Holzer, A. K., Safaei, R., & Howell, S. B. (2004). Modulation of the cellular pharmacology of cisplatin and its analogs by the copper exporters ATP7A and ATP7B. *Mol Pharmacol*, 66(Jul), 25-32.

Samimi, G., Safaei, R., Katano, K., Holzer, A. K., Rochdi, M., Tomioka, M., Goodman, M., & Howell, S. B. (2004). Increased expression of the copper efflux transporter ATP7A mediates resistance to cisplatin, carboplatin and oxaliplatin in ovarian cancer cells. *Clin Cancer Res*, 10(14), 4661-4669.

Samimi, G., Varki, N. M., Wilczynski, S., Safaei, R., Alberts, D. S., & Howell, S. B. (2003). Increase in expression of the copper transporter ATP7A during platinum drug-based treatment is associated with poor survival in ovarian cancer patients. *Clin Cancer Res*, 9(16), 5853-5859.

Scott, D. C., & Schekman, R. (2008). Role of Sec61p in the ER-associated degradation of short-lived transmembrane proteins. *J Cell Biol*, 181(7), 1095-1105.

Setty, S. R., Tenza, D., Sviderskaya, E. V., Bennett, D. C., Raposo, G., & Marks, M. S. (2008). Cell-specific ATP7A transport sustains copper-dependent tyrosinase activity in melanosomes. *Nature*, 454(7208), 1142-1146.

Suzuki, M., & Gitlin, J. D. (1999). Intracellular localization of the Menkes and Wilson's disease proteins and their role in intracellular copper transport. *Pediatr Int*, 41(4), 436-442.

Toikkanen, J., Gatti, E., Takei, K., Saloheimo, M., Olkkonen, V. M., Soderlund, H., De Camilli, P., & Keranen, S. (1996). Yeast protein translocation complex: isolation of two genes SEB1 and SEB2 encoding proteins homologous to the Sec61 beta subunit. *Yeast*, 12(5), 425-438.

Toikkanen, J. H., Miller, K. J., Soderlund, H., Jantti, J., & Keranen, S. (2003). The beta subunit of the Sec61p endoplasmic reticulum translocon interacts with the exocyst complex in *Saccharomyces cerevisiae*. *J Biol Chem*, 278(23), 20946-20953.

Van den Berg, B., Clemons, W. M., Jr., Collinson, I., Modis, Y., Hartmann, E., Harrison, S. C., & Rapoport, T. A. (2004). X-ray structure of a protein-conducting channel. *Nature*, 427(6969), 36-44.

Wu, C., Orozco, C., Boyer, J., Leglise, M., Goodale, J., Batalov, S., Hodge, C. L., Haase, J., Janes, J., Huss, J. W., 3rd, & Su, A. I. (2009). BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources. *Genome Biol*, 10(11), R130.

Zhao, X., & Jantti, J. (2009). Functional characterization of the trans-membrane domain interactions of the Sec61 protein translocation complex beta-subunit. *BMC Cell Biol*, 10, 76.

FOOTNOTES

This work was supported by grants from the National Cancer Institute and National Institutes of Health [Grants T32CA121938, CA152185 and CA095298], as well as by a grant from the Tower Cancer Research Foundation. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health.

FIGURE LEGENDS

Figure 1. Characterization of 2008 Sec61 β KD cells. (A) Relative Sec61 β mRNA levels in parental and 2008 Sec61 β KD cells as measured by qRT-PCR. (B) Western blot analysis of Sec61 β protein level. Vertical bars, \pm SD. Concentration-survival curves of parental 2008 WT (—◇—) and 2008 Sec61 β KD cells (---■---) for: (C) cDDP; (D) carboplatin; and (E) oxaliplatin as measured by SRB assay. Survival plotted on log scale. Vertical bars, \pm SEM.

Figure 2. Effect of Sec61 β knockdown on Pt accumulation. (A) Whole cell Pt accumulation following 1 h exposure to 30 μ M cDDP as measured by ICP-MS in 2008 WT (■) and 2008 Sec61 β KD (□) cells. (B) The extent of DNA-Pt adduct formation following same treatment as in (A). Vertical bars, \pm SEM.

Figure 3. Analysis of the expression proteins involved in Cu homeostasis. (A) qPCR analysis of the effect of Sec61 β knockdown on mRNA expression of proteins involved in Cu homeostasis in 2008 WT (■) and 2008 Sec61 β KD (□). Vertical bars, \pm SEM. (B) Western blot analysis of the effect of Sec61 β knockdown. Blots are representative of a minimum of three independent experiments. (C) Western blot analysis of effect of cycloheximide on ATP7A stability. The blot is representative of three independent experiments. (D) Bands quantified and normalized to actin intensity. 2008 WT (—◆—) and 2008 Sec61 β KD (·□·). Vertical bars, \pm SEM.

Figure 4. Effect of Sec61 β knockdown on Cu accumulation and sensitivity. (A) Basal Cu content and level of Cu accumulation following exposure to 100 μ M CuSO₄. (B) Cu concentration-survival curves of parental 2008 WT (—◇—) and 2008 Sec61 β KD cells (---■---). Survival plotted on log scale. Vertical bars, \pm SEM.

Figure 5. Western blot analysis of the effect of Cu, BCS and cDDP on ATP7A expression in the parental 2008 and 2008 Sec61 β KD cells. Cell lysates were prepared from 2008 wild type and 2008 Sec61 β KD cells following a 1 h incubation with 200 μ M CuSO₄, 100 μ M BCS, or 30 μ M cDDP, and western blots probed with the antibodies against the proteins indicated.

Figure 6. Effect of Sec61 β knockdown in Me32a human fibroblasts lacking ATP7A. (A) Western blot of ATP7A and Sec61 β in Me32a and Me32a Sec61 β KD cells. (B) qPCR analysis of the effect of Sec61 β knockdown on mRNA expression of ATP7A (■) and Sec61 β (□) in Me32a and Me32a Sec61 β KD cells. Vertical bars, \pm SEM. (C) cDDP concentration-survival curves of parental Me32a (—◇—) and Me32a Sec61 β KD cells (---■---). Survival plotted on log scale. Vertical bars, \pm SEM.

Figure 7. Deconvolution microscopic analysis of 2008 wild type and 2008 Sec61 β KD cells. Cells were fixed and stained for ATP7A, the trans-Golgi marker syntaxin-6, and nuclear stain Hoechst 33342 following a 1 h treatment with 100 μ M BCS or 200 μ M CuSO₄.

Figure 8. Lipid raft fractionation of ATP7A and Sec61 β in 2008 cells. Sucrose gradient centrifugation was performed for 2008 cells. Eleven fractions from the top (1-11) and a total lysate sample (L) were isolated and assessed by western blot using the antibodies indicated.

Figure 9. Cell surface expression of ATP7A in response to copper by cell surface biotinylation in the parental 2008 and 2008 Sec61 β KD cells. Cells were left untreated or were incubated for 1 h with 200 μ M CuSO₄, after which cell surface proteins were biotinylated and pulled down with immobilized streptavidin and assessed by western blot. β -actin and Na/K ATPase were probed as controls to confirm quality of sample preparation.

Table 1. IC₅₀ (μM) values ^a for various chemotherapeutic agents and copper.

Drug	Wild type 2008	2008 Sec61β KD	Fold increase	P - value
Cisplatin	2.60 ± 0.62	21.63 ± 4.88	8	< 0.001
Carboplatin	28.84 ± 0.63	462.13 ± 11.36	16.8	< 0.001
Oxaliplatin	0.33 ± 0.02	2.49 ± 0.30	9.6	< 0.01
Copper	36.05 ± 0.05	166.02 ± 0.08	5	< 0.01
Etoposide	1.05 ± 0.16	7.50 ± 2.29	7	< 0.05
Vincristine	0.0067 ± 0.0004	0.0129 ± 0.002	2.1	< 0.01
Doxorubicin	0.074 ± 0.036	0.221 ± 0.093	3.1	< 0.01
Paclitaxel	0.0020 ± 0.0002	0.0034 ± 0.0002	1.7	< 0.001

^a Mean ± SEM

Figure 1

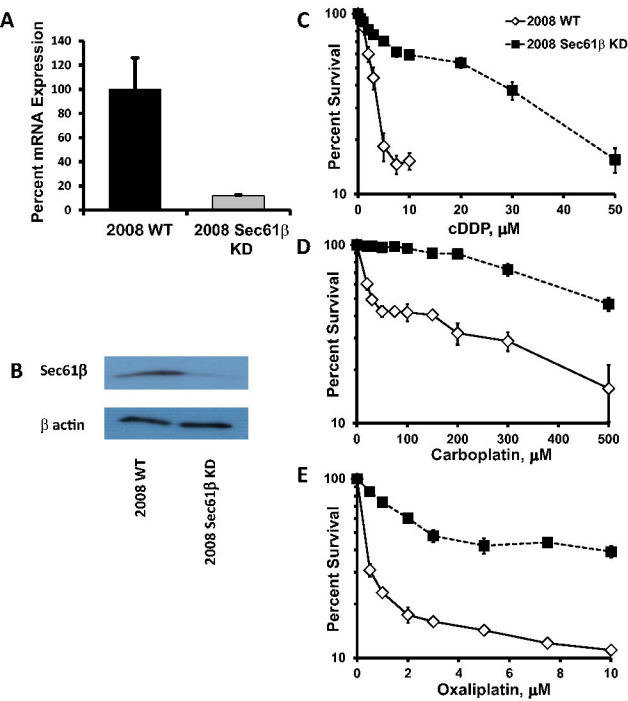


Figure 2

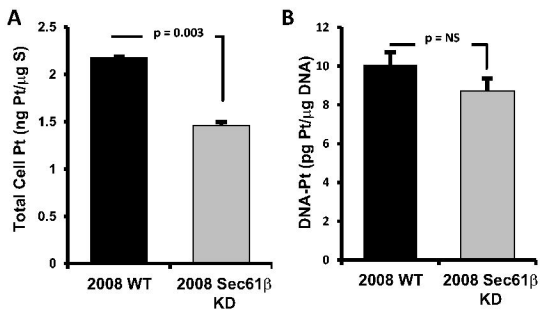


Figure 3

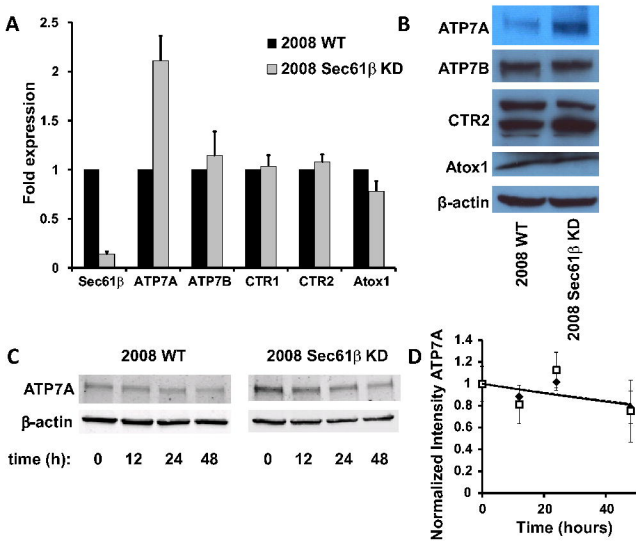


Figure 4

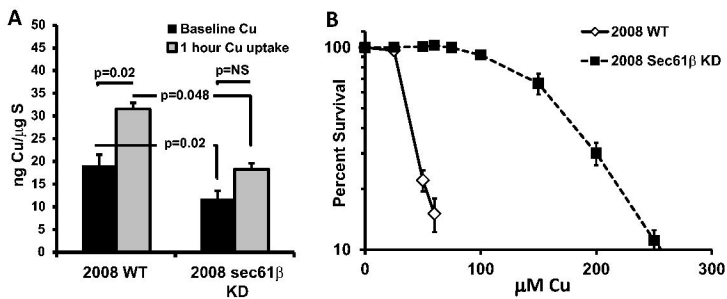


Figure 5

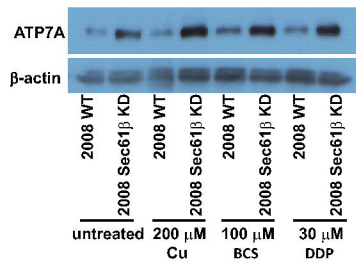
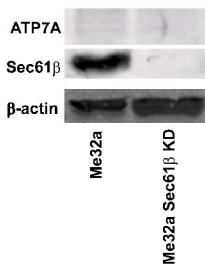
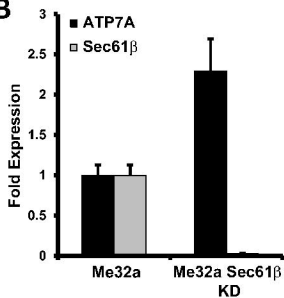


Figure 6

A



B



C

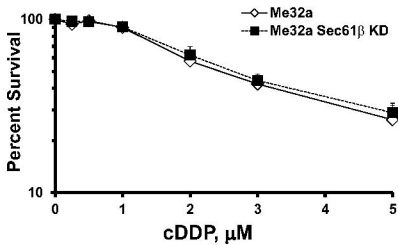


Figure 7

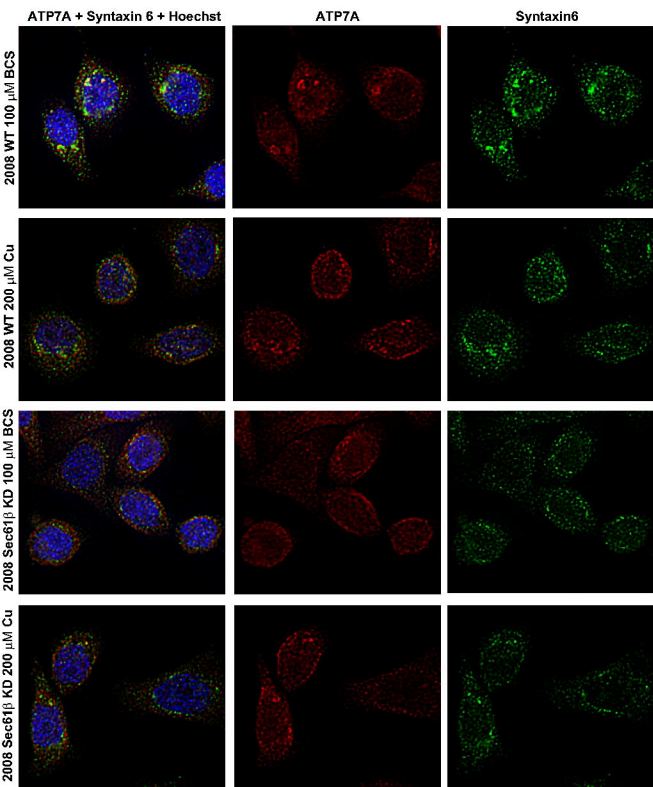


Figure 8

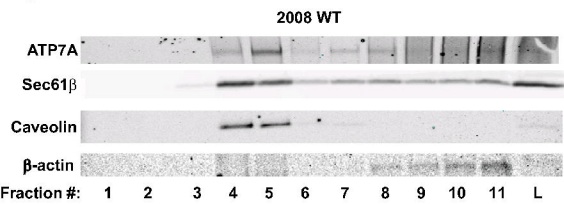


Figure 9

