Chloroquine Promotes Apoptosis in Melanoma Cells by Inhibiting BH3 domain Mediated PUMA Degradation

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Abstract

The BH3-only protein PUMA counters Bcl-2 family anti-apoptotic proteins and promotes apoptosis. Although PUMA is a key regulator of apoptosis, the post-transcriptional mechanisms that control PUMA protein stability are not understood. We show that a lysosome-independent activity of chloroquine prevents degradation of PUMA protein, promotes apoptosis and reduces the growth of melanoma xenografts in mice. Compared to wild–type PUMA, a BH3 domain deleted PUMA protein showed impaired decay in melanoma cells. Fusion of the BH3 domain to a heterologous protein led to its rapid turnover that was inhibited by chloroquine. While both chloroquine and inhibitors of lysosomal proteases stalled autophagy, only choroquine stabilized PUMA protein and promoted apoptosis. Our results reveal a lysosomal protease independent activity of chloroquine that selectively promotes apoptosis in melanoma cells.

Introduction

Melanoma is one of the most aggressive cancers that presents a great clinical challenge to treat. While primary melanoma is curable by early surgical excision, metastatic melanomas are highly aggressive with an average survival of 6–10 months (Tsao *et al.*, 2012). Despite a large number of clinical trials and considerable progress in understanding the molecular etiology, metastatic melanoma remains resistant to treatment and the lifetime risk of it continues to steadily increase (Gray-Schopfer *et al.*, 2007). Recently, BRAF and MAPK inhibitors have shown significant clinical activity, yet these successes are limited by the swift development of resistance (Ji *et al.*, 2012). Identification of novel therapeutic targets and additional treatment options are much needed to combat this malignancy.

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Apoptosis is a cellular demolition process that involves an intricate interplay of pro- and anti-apoptotic Bcl-2 family proteins leading to mitochondrial damage and cytochrome C release followed by caspase activation (Chipuk *et al.*, 2010; Green and Evan, 2002). Resistance to apoptosis plays a central role in tumor progression and enables malignant cells to become unresponsive to chemotherapy and radiation treatment modalities (Green and Evan, 2002). Increased production of anti-apoptotic proteins or silencing of pro-apoptotic proteins contribute to impaired apoptosis. Bcl-2 family anti-apoptotic members consist of Bcl-2, Bcl-Xl, Mcl-1 and Bcl-w, and pro-apoptotic members include Bim, Bid, Bad, Bak, Bax, NOXA and PUMA (Chipuk *et al.*, 2008; Chipuk and Green, 2008; Chipuk *et al.*, 2010). The pro-apoptotic proteins harbor a single BH3 domain and are called as BH3-only proteins. The BH3 domain of these proteins contains an amphipathic α-helix structure that engages the hydrophobic groove of pro-survival partners to activate Bak and Bax at the mitochondria (Sattler *et al.*, 1997).

The levels of BH3-only proteins are tightly regulated to restrict cell destruction (Strasser *et al.*, 2011). Expression of these proteins is regulated by various mechanisms including enhanced transcription and post-translations modifications (Puthalakath and Strasser, 2002). The best example of transcriptional control of BH3-only proteins derives from studies of p53-mediated transcriptional targets. In response to DNA damage, p53 binds to specific sequence elements near the promoters of PUMA and NOXA genes and enhances their transcription (Nakano and Vousden, 2001; Yu *et al.*, 2001). As a result, pro-apoptotic BH3-only protein levels increase and result in mitochondrial permeabilization with subsequent activation of caspase-mediated apoptosis. Tumor cells avoid this cell death mechanism by inactivating upstream components of p53 or selecting for p53 mutations (Vousden and Lane, 2007; Vousden and Prives, 2009). While mutations in p53 gene are common in multiple malignancies, they are rare in melanoma (Chin *et al.*, 1998; Lee and Herlyn, 2012). The upstream regulator of p53 called p14ARF is mutated in familial melanoma (Chin *et al.*, 1998). Perturbation of downstream components of the apoptotic pathway provides a selective advantage in circumventing the need for inactivating mutations of the p53 gene. For example, expression of the downstream apoptotic factor Apaf-1 is silenced in some melanomas (Soengas *et al.*, 2001). These studies support the notion that both upstream and downstream components of p53 pathways are disabled in melanoma.

Autophagy is a regulated degradative process that recycles damaged organelles and cytoplasmic macromolecules to enable cell survival (Levine and Kroemer, 2008). Typically, autophagy involves sequestration of cytoplasmic components in a double membrane, the contents of which are degraded by delivery to the lysosome. Chloroquine (CQ) is a classical anti-malarial and anti-inflammatory drug that inhibits lysosomal acidification. In cell culture assays, CQ is commonly used as an inhibitor of autophagy. Depending on the type of cancer, autophagy may become dysregulated in a manner that enables tumor cell survival even in a limited nutrient environment (Baehrecke, 2005; White, 2012). This implication led to the use of CQ as a small molecule inhibitor of autophagy in xenograft tumor studies and clinical trials (Amaravadi *et al.*, 2011b; Sheen *et al.*, 2011; Tormo *et al.*, 2009; Yang *et al.*, 2011). While CQ has also been reported to promote apoptosis in several tumor models (Amaravadi *et al.*, 2007; Boya *et al.*, 2005; Maclean *et al.*, 2008; Tang *et al.*, 2011; Walls *et*

al., 2010), its effects on PUMA remain unclear. Here we use CQ to reveal the BH3 domain-dependent and lysosome-independent degradation of PUMA protein and associated apoptosis in melanoma cells.

Results

Chloroquine activates apoptosis in melanoma cells

Because CQ exerts anti-tumor activity (Amaravadi *et al.*, 2011b; Amaravadi *et al.*, 2007; Sheen *et al.*, 2011), we examined cell survival of human melanoma derived cell lines (SK-MEL23, MEL501, MELSK5, MEL526 and MEL624) and non-melanoma cell lines (HCT116, MCF7 and H1299) after treatment with chloroquine. Based on pilot studies with a range of concentrations, we chose 50μM chloroquine for further experiments. Viability of the majority of melanoma cell lines decreased with 24 hours of exposure to 50μM chloroquine (Fig 1a). In contrast, viability of the non-melanoma cell lines was largely unchanged. We then measured caspase activity as an indicator of apoptosis. The decreased viability of melanoma cells correlated with a dramatic increase in caspase levels, which was not observed in the non-melanoma cell lines (Fig 1b).

Oncogenic mutations in BRAF and N-RAS are known to promote melanomagenesis (Dankort *et al.*, 2009; Davies *et al.*, 2002; Pollock *et al.*, 2003). To explore the relevance of these upstream signaling pathways in chloroquine-induced apoptosis, we used human melanoma cell lines that express wild type BRAF and N-RAS genes (SK-mel187, mel505, RPMI8322), cells mutant for N-RAS (SK-mel103, mel224, VMM39), and cell lines with BRAF mutations (SK-mel24, SK-mel28, WM2664). All melanoma cell lines with the exception of SK-mel187 showed decreased viability and a corresponding increase in caspase activity in response to the CQ treatment (Fig 1c,d). Therefore, there was no correlation between CQ responsiveness and the oncogenic mutation in these melanoma cell lines.

Chloroquine inhibits melanoma tumor growth *IN VIVO*

To test the effects of CQ in an *in vivo* model, we implanted SK-MEL23 melanoma cells in NOD-SCID mice and evaluated the effects of CQ on subcutaneous tumor growth. Melanoma cells were allowed to establish an average volume of 100 mm^3 at which point the mice were divided into two groups. A set of four mice served as vehicle control and six mice were treated with a low dose of CQ (25 mg/kg body weight) on an every three-day treatment schedule for 21 days. Tumor volume measurements showed that chloroquine treatment reduced the tumor volume in the experimental group (p = 0.0001) compared to the vehicle controls (Fig 2a). There were no differences in total body weight between the treatment and control groups. At the end of the study, xenografts were excised and gross weight values of tumors were recorded (Fig 2b). Consistent with tumor volume measurements, we found a statistically significant (p=<0.05) reduction in tumor mass from this low dose of CQ-treated animals as compared to the vehicle group.

Chloroquine promotes PUMA protein accumulation in melanoma cells

Chloroquine-induced activation of apoptosis led us to study its effects on expression of the pro-apoptotic protein PUMA. SK-MEL23 and VMM39 melanoma cells were treated with

etoposide as an activator of p53-mediated PUMA induction (Nakano and Vousden, 2001; Yu *et al.*, 2001) or with different concentrations of chloroquine (25 and 50μM). Immunoblot analysis of these cell extracts showed that etoposide induction of p53 increased the levels of its target proteins PUMA and p21 (Fig 3a,b). Remarkably, chloroquine also increased levels of PUMA protein in a dosage-dependent manner while the levels of p53 and p21 did not change. In an effort to address the requirement for PUMA in CQ-mediated apoptosis, SK-MEL23 cells were transfected with control or PUMA siRNAs and exposed to CQ. The results showed that siRNA efficiently depleted PUMA protein and this resulted in dramatic reduction of CQ-mediated apoptosis (Suppl Fig S1). To substantiate these results, we treated additional melanoma cell lines (mel526 and mel501) with chloroquine and observed similar effects on PUMA (Suppl Fig S2). Although PUMA protein was induced in response to p53 activation (Fig 3a,b), chloroquine did not change the levels PUMA protein in MCF7 breast cancer cells (Suppl Fig S3) and correspondingly there was no apoptotic response (Fig 1b). To test whether the observed increase in PUMA protein levels was due to transcription, we treated several melanoma cell lines with CQ and quantified PUMA transcripts. The results showed that CQ did not change the transcript levels of PUMA (Fig 3d,e).

To demonstrate that the CQ-mediated increase in the levels of endogenous PUMA protein is due to inhibition of its degradation, we determined the stability of PUMA protein in the presence or absence of CQ. Cycloheximide is a *de novo* protein synthesis inhibitor commonly used to evaluate the half-life of proteins (Ballard, 1977; Carreira *et al.*, 2006; Waters *et al.*, 1991). SK-MEL23 melanoma cells were treated with cycloheximide in the presence or absence of CQ and the cell extracts were blotted for PUMA protein (Fig 4a,b). PUMA protein signal intensity from three independent experiments was quantified by luminescence measurements (Fig 4c). There was a 50% decrease of endogenous PUMA protein in 3 hours in the absence of CQ. Chloroquine prolonged the stability of this protein and even after 5 hours of cycloheximide exposure nearly 80% of PUMA protein remained.

BH3-domain is essential for PUMA protein degradation

The BH3 domain of PUMA engages the hydrophobic groove of anti-apoptotic family members to activate apoptosis. Because this domain is critical for apoptosis, we reasoned that it may be involved in PUMA destabilization and that a mechanism in melanoma cells specifically may target this domain to avoid cell death. To test this idea, we introduced plasmids encoding for full-length or BH3-deletion mutant PUMA into melanoma cells and conducted cycloheximide chase assays for these ectopically expressed proteins. After 5 hours of cycloheximide incubation, nearly 50% of full-length PUMA protein remained (Fig 5a,b), while the BH3 domain deletion mutant protein levels were unchanged. These results suggest that the BH3 domain plays a critical role in PUMA protein destabilization. We questioned whether this BH3 domain was sufficient to direct degradation of a heterologous protein. We constructed GFP fusions with a BH3 domain or a mutant BH3 domain (mBH3) devoid of the conserved amino acid sequence Leu-Arg-Arg (Fig 5c). We introduced these recombinant plasmids into melanoma cells and examined GFP expression. No GFP signal was visualized with the wild-type BH3 fusion transfected cells (Fig 5d). In contrast, the mutant BH3 domain showed intense GFP fluorescence (Fig 5d). To further determine the effects of CQ on BH3 fusion protein stability, melanoma cells were transfected with these

GFP constructs and treated with chloroquine. Immunoblots for GFP showed that chloroquine treatment restored the levels of wild-type BH3 domain fused GFP (Fig 5e). These results reinforce the essential role of the BH3 domain in PUMA degradation and that CQ inhibits degradation of PUMA actuated by this domain.

Because CQ did not elicit an apoptotic response and did not stabilize PUMA protein in non-melanoma cell lines, we questioned the integrity of this BH3 targeting mechanism in these cell lines. When GFP fusion constructs were transduced into non-melanoma cell lines (MCF7, H1299 and HCT116) similar GFP fluorescence intensities were observed for both wild-type and mutant BH3 fusion proteins (Suppl Fig S4).

Lysosomal protease-independent degradation of PUMA protein

Chloroquine is generally considered to exert its drug effect through inhibition of lysosomal acidification. Because melanoma cells treated with CQ increased the levels of PUMA protein, we explored whether a lysosomal process causes PUMA protein destabilization. Surprisingly, bafilomycin A, a commonly used lysomotropic compound, and the lysosomal cathepsin inhibitor ALLN had no affect on PUMA protein levels (Suppl Fig S5). Furthermore, the protease inhibitor leupeptin and proteasome inhibitors lactacystin did not inhibit degradation of PUMA protein (Suppl Fig S5). MG132 treatment produced a slight increase in the PUMA protein levels, but not nearly as much as CQ (Suppl Fig S5). We reasoned that this is most likely due to robust p53 protein stabilization by MG132. Although ALLN, MG132 and lactacystin increase levels of p53, PUMA protein degradation was specifically inhibited by chloroquine. We also incubated melanoma cells with either CQ or the combination of lysosomal protease inhibitors E64d and pepstatin A (Tanida *et al.*, 2005). The increase in PUMA was exclusive to CQ, although both treatments strongly inhibited lysosomal activity as assessed by the autophagy markers LC3 and p62 (Fig 6a,b,c,d). Consistent with their effects on PUMA, CQ but not the lysosomal inhibitors stimulated apoptosis (Fig 6e). These results clearly demonstrate that lysosomal protease function is dispensable for PUMA degradation in melanoma cells.

Discussion

Impaired apoptosis is one of the critical steps in malignant transformation. Understanding this pathway in tumor cells may have major clinical implications, as chemoresistant malignancies are generally unable to activate apoptotic responses. Here we show that chloroquine has a profound effect on melanoma cell viability and promotes apoptosis. Although chloroquine did not alter the levels of p53 protein, it caused a transcription-independent increase in levels of PUMA protein and promoted apoptosis. We demonstrate that chloroquine increases the half-life of PUMA protein in multiple human melanoma-derived cell lines. In these cells, the BH3 domain of PUMA protein is responsible for its destabilization. Remarkably, the BH3 domain was sufficient to promote degradation of a heterologous protein and this was selective for melanoma cells. Furthermore, we demonstrate that chloroquine-mediated inhibition of PUMA protein degradation does not depend on lysosomal proteases. Because the BH3 domain is critical for pro-apoptotic

function for the protein, melanoma cells recognize this death promoting peptide region and degrade PUMA to avoid cell death.

Macroautophagy is commonly referred to as autophagy. In response to nutrient depletion, activation of autophagy leads to digestion of cytosolic macromolecules that provides energy sources required for cell survival. Importantly, oncogenic RAS transformation relies on autophagy to maintain energy balance (Guo *et al.*, 2011; Lock and Debnath, 2011; Marino *et al.*, 2011). This represents an advantage for malignant cells to multiply in a microenvironment with limited nutrient supplies. Therefore, targeting this autophagy dependency is a rational approach for anti-tumor therapy (Amaravadi *et al.*, 2011a). For example, inhibition of autophagy by chloroquine led to selective pancreatic tumor regression and prolonged survival of mice implanted with pancreatic cancer xenografts (Yang *et al.*, 2011). HeLa cells exposed to the autophagy inhibitors hydroxychloroquine, bafilomycin A1, and monensin displayed increased mitochondrial cell death (Boya *et al.*, 2005). In our study, and consistent with the observations from other reports (Guo *et al.*, 2011; Yang *et al.*, 2011), chloroquine exposure did not elicit caspase-mediated apoptotic responses in non-melanoma cell lines MCF7, HCT116 and H1299. Importantly, the majority of the melanoma cells dramatically activated apoptosis upon treatment with CQ. This led us to investigate the role of the p53-PUMA pathway that has been documented to initiate caspase-dependent apoptosis. Surprisingly, CQ treatment of melanoma cells resulted in PUMA protein accumulation but there was no increase in p53 protein. It is relevant to note that PUMA transcription is regulated by p53 dependent and independent mechanisms (Gomes and Espinosa, 2010; Schumm *et al.*, 2006; Sykes *et al.*, 2006; Tang *et al.*, 2006; Wang *et al.*, 2009). Quantitative transcript analysis of CQ-treated melanoma cells showed that PUMA transcription was unchanged. Furthermore, protein stability studies demonstrate that CQ inhibits proteolysis of PUMA. PUMA protein undergoes phosphorylation at multiple sites, particularly serine 10, which leads to its destabilization (Fricker *et al.*, 2010). It is unlikely that CQ impacts this phosphorylation as we demonstrate CQ stabilization is dependent on the BH3 domain of PUMA. It is unlikely that degradation of PUMA protein involves the proteasome for the following reasons: the absence of lysines for ubiquitin conjugation in PUMA and chloroquine promoted PUMA protein stability in a lysosomal activity-independent manner. It is likely that CQ can stabilize other BH3-domain proteins. However, unlike PUMA, which lacks lysine, NOXA contains multiple lysines that may facilitate ubiquitin-mediated proteasomal degradation even in the presence of CQ.

Chloroquine is widely used to treat malaria by halting the *P. falciparum* replication cycle (Cooper and Magwere, 2008). CQ acts as a weak base that neutralizes low pH in the lysosome, thereby inactivating lysosomal proteases. We demonstrate an activity of CQ that is distinct from its classical function of lysosomal protease inhibition. CQ blocked degradation of PUMA, which requires the BH3 domain. This inhibition cannot be mediated by ubiquitination of PUMA nor dependent on lysosomal proteases, so the mechanism remains to be determined. The mechanism may be unique to melanoma, as we have not observed CQ-associated PUMA protein stability in several non-melanoma cell lines. Further investigation on how CQ alters the catabolism of BH3 proteins such as PUMA may lead to novel drug targets. Acidic organelles are generally sensitive to CQ exposure as it is a weak

base that neutralizes their pH. This property of CQ led us to suspect melanosomes as a possible site of PUMA protein degradation. Lysosomal protease inhibitors treatment increased the levels of the melanosome protein PMEL17 to a larger extend than CQ exposure (Suppl Fig S6), suggesting that PUMA may not be degraded in melanosomes.

Given the limited treatment options for melanoma, these studies suggest that melanoma may respond to a blockade of the mediators of BH3-dependent PUMA turnover. Consistent with our findings, chloroquine reduced melanoma tumor growth *in vivo*. Further evidence is suggested by the observation that leucine deprivation combined with chloroquine exposure led to widespread apoptosis in human melanoma cells (Sheen *et al.*, 2011). High-dose IL-2 is currently a treatment option for advanced stage melanoma. However, the toxicity associated with IL-2 administration has been problematic. Recently, IL-2 was combined with chloroquine to demonstrate inhibition of metastatic tumor growth in a murine model (Liang *et al.*, 2012). The present studies provide a molecular basis for the anti-melanoma effect of CQ. Combined with our demonstration of CQ-associated PUMA activation, we propose that chloroquine may be useful in combination therapy for metastatic melanoma. Moreover, CQ might exert synergistic effects with chemotherapeutic agents (e.g., temozolomide) or BRAF-inhibitors (Bollag *et al.*, 2012) in the treatment of melanoma. Although our *in vivo* studies show moderate tumor growth inhibition in the presence of 25mg/kg of CQ, the concentration of CQ used in our *in vitro* experiments are higher than that used clinically. Additional animal studies are needed to translate the use of CQ in combination therapy to man.

Materials and Methods

Cell culture and reagents

HCT116 and MCF7 cells were maintained in high-glucose DMEM medium (Invitrogen) supplemented with L-glutamine and sodium pyruvate, with 10% fetal bovine serum (FBS) (Sigma). H1299 and melanoma cell lines (SK-MEL23, MEL-501, MEL-526, MEL-624, and MEL-697) were maintained in RPMI media (Invitrogen) with 10% FBS (Sigma). Bafilomycin A, chloroquine diphosphate, leupeptin, and MG-132 were obtained from Sigma, ALLN, etoposide, and Z-Vad-Fmk from R&D Systems (Minneapolis), E64d and Pepstatin A (Cayman Chemical) and lactacystin (AG Scientific).

Transfection

Human BH3 domain and the BH3 domain mutant lacking the LRR motif were cloned into EGFP vector, to express GFP-fusion proteins. Cells were grown to 50–60% confluency in 6-well culture plates (Corning) with collagen-1 coated coverslips (BD Biosciences). All melanoma cell lines were transfected with FuGENE HD (Promega) according to manufacturer's protocol. HCT116, MCF7, and H1299 cells were transfected with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol.

Whole-cell extracts and western blot analysis

Whole-cell extracts were prepared in urea buffer (6M urea, 100mM sodium dihydrophosphate, 10mM Tris pH8). SDS-PAGE was performed using TGX gradient gels

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(Bio-Rad) and transferred onto PVDF (Millipore) using TransBlot SD semi-dry transfer apparatus (BioRad) as per manufacturer's guidelines. The blots were probed with following antibodies; GFP, p53 & p21 (Santa Cruz), LC3 (Novus Biologicals), NOXA (Pierce), PUMA (AnaSpec), actin and p62 (Sigma). Blot images were captured on ImageQuant LAS 4000 digital imaging system (GE Healthcare).

Apoptosis and viability assays

The cells (10×10^3/each well) were plated in 96-well plates (Corning). After culture overnight, either fresh media (control) or fresh media containing 50 uM of CQ was added for indicated time periods. The activation of apoptosis was determined using Caspase-Glo 3/7 Assay System according to the manufacturer's instructions (Promega). Cell viability was assessed using MTS-based assay (Promega).

In-vivo tumor implantation

NOD-SCID mice were implanted with 1.5 million of human melanoma SKMel23 cells in 100μl serum free media subcutaneously into the right hind flank. Tumors were allowed to develop for 21 days until they reached average palpable size of 100 mm^3. At this point, the animals were randomly divided into two groups, four controls and six treatment subjects. The control group received intraperitoneal injections of sterile saline (50μl), and treatment group was similarly injected with CQ at the concentration of 25mg/kg in saline, twice a week for 3 weeks. Xenograft size was measured twice a week with a digital caliper and the ellipsoidal tumor volumes were recorded prior to administration of each placebo or treatment injection. The Animal Care and Use Committee approved all procedures used in these experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

CQ	chloroquine
PUMA	p53 Upregulated Modulator of Apoptosis
wt	wild type
BH	Bcl homology

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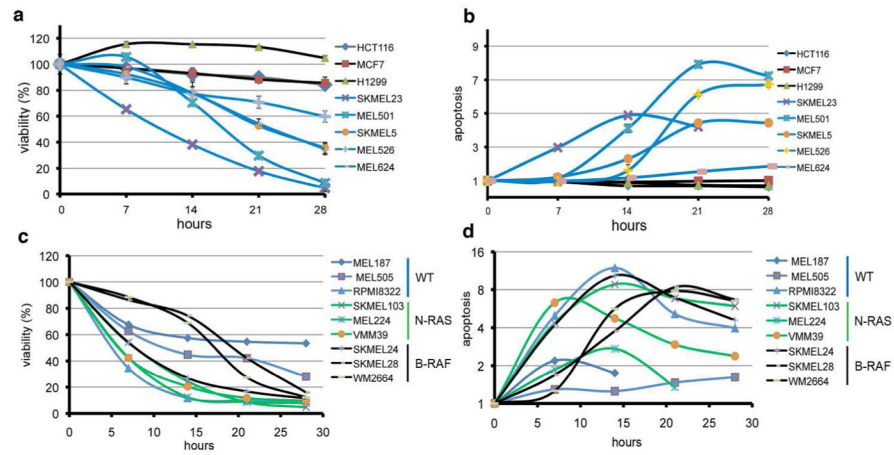
Figure 1. Chloroquine promotes apoptosis in melanoma cell lines
Three non-melanoma and five melanoma cell lines were exposed to 50IM CQ, cell viability (a) and apoptosis (b) were measured at indicated time points. c & d) Wild-type for N-RAS and BRAF or its mutant melanoma cell lines were treated with CQ as in A & B and the cell viability (c) and apoptotic response (d) to CQ was assessed.
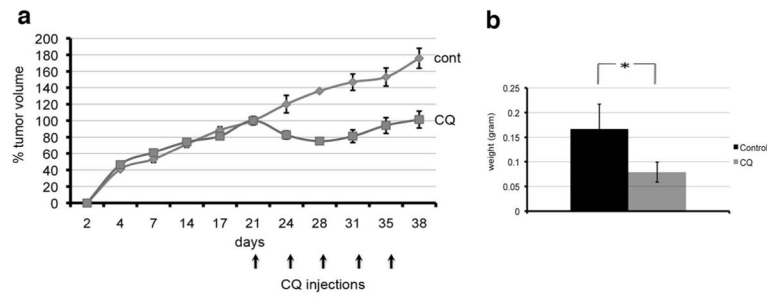
Figure 2. Chloroquine reduces growth of human melanoma cells in mice
a) NOD-SCID mice were implanted with SK-MEL23 cells, 21 days post-implantation, mice were divided in to two groups for vehicle and CQ treatment. Tumor volumes of vehicle or CQ treated mice were measured at indicated days and the growth kinetics of tumor volume shown on the graph (p = 0.0001). b) Dissected tumors from vehicle and CQ treated mice were weighed (p=<0.05).
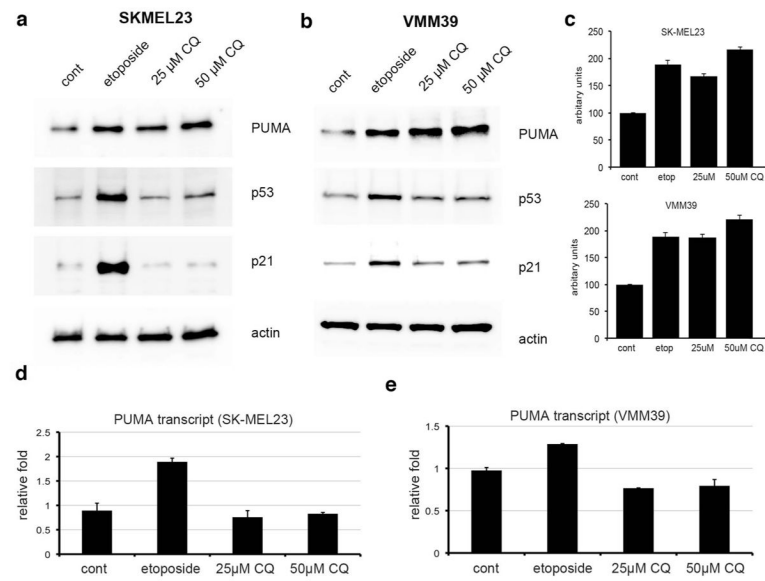
Figure 3. CQ promotes accumulation of PUMA protein in melanoma cells
a & b) Indicated melanoma cells were exposed to etoposide or different concentrations of CQ and the cell extracts were blotted with PUMA, p53, p21 and actin antibodies. c) Shows quantification of PUMA signal intensity from a & b. d & e) Melanoma cells were treated with etoposide or CQ and the relative levels of PUMA transcripts were assayed by q-PCR.
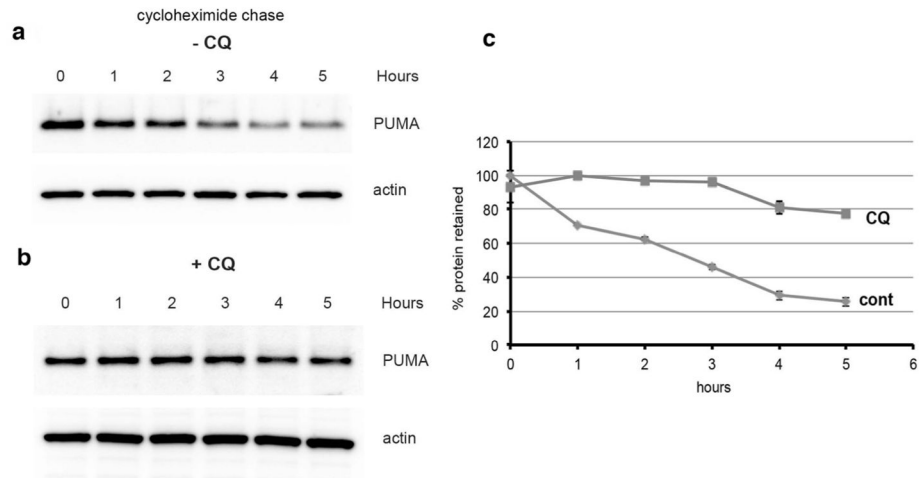
Figure 4. CQ inhibits degradation of PUMA protein in melanoma cells
a & b) To determine the half-life of endogenous PUMA protein, control or CQ treated melanoma cells were exposed to cycloheximide, a protein synthesis inhibitor, and cell extracts harvested at different time points were blotted with PUMA antibody. c) Protein decay analysis from three experiments shown.
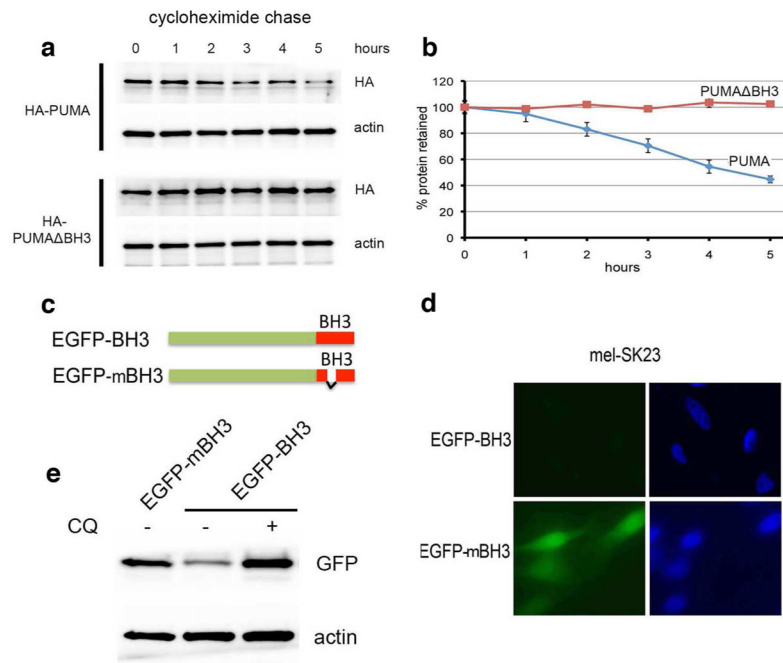
Figure 5. The BH3 domain of PUMA mediates its degradation
a) To determine the role of the BH3 domain in PUMA protein stability, recombinant plasmids coding for HA-tagged wild-type or BH3 domain-deletion mutant PUMA were ectopically expressed in melanoma cells and the cell extracts were blotted with HA antibody or actin antibodies. b) Densitometry scanning of three experiments is shown on a graph. c) Schematic showing BH3 domain and its mutant peptides fusion with GFP. d) Plasmids from c were introduced into melanoma cells and the GFP expression were imaged. e) Mutant BH3 domain and wt-BH3 domain fused to GFP expressed in melanoma cells and the cells treated with CQ and blotted with indicated antibodies.
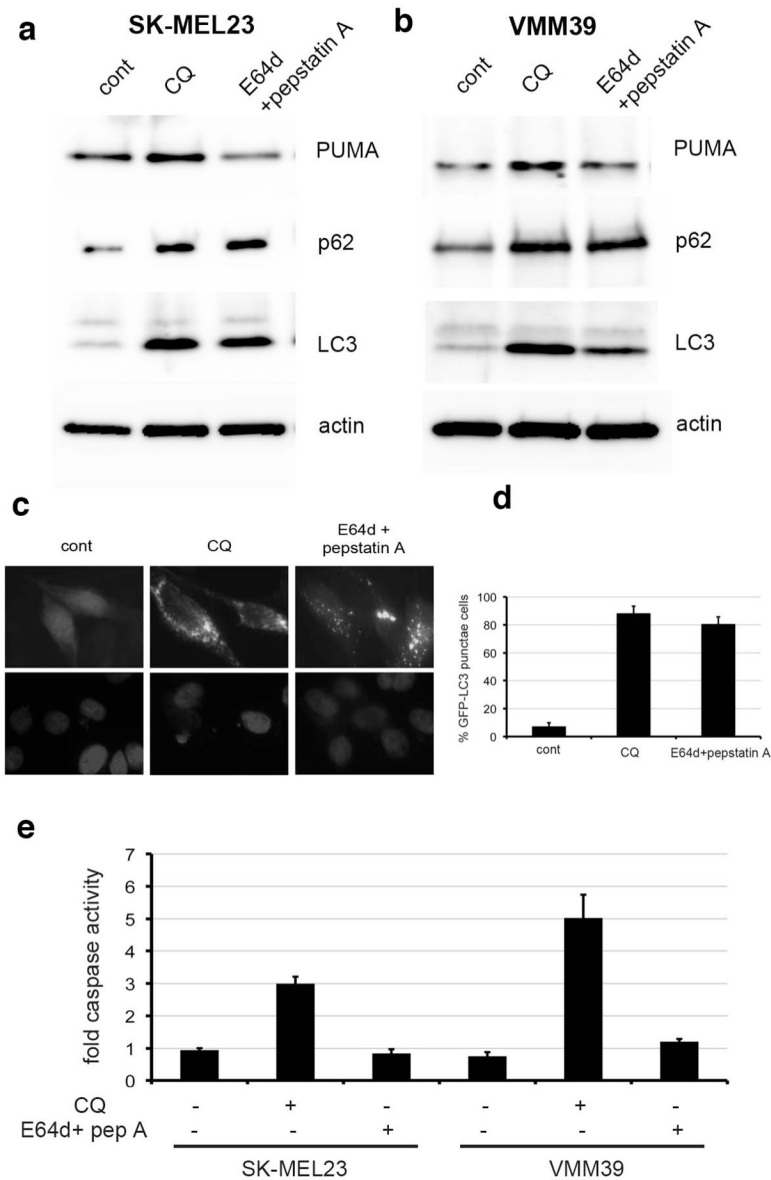
Figure 6. Lysosomal protease-independent degradation of PUMA protein in melanoma cells
a & b) Melanoma cells were exposed to CQ or lysosomal protease inhibitor cocktails (E64d
+pepstatin A) and the cell extracts were blotted with antibodies shown. c) Melanoma cells
transfected with GFP-LC3, exposed to CQ or lysosomal protease inhibitor cocktails and
visualized under microscope. d) GFP punctae counted from three experiments show on
graph. e) Melanoma cells treated with CQ or lysosomal protease inhibitors and the measured
caspase activity shown.