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AUTHOR(S)

Karen Boland, Lorna Flanagan, Niamh McCawley, Ritesh Pabari, Elaine W. Kay, Deborah A. McNamara, Frank Murray, Annette Byrne, Zebunnissa Ramtoola, Caoimhín Concannon, Jochen Prehn

CITATION

Boland, Karen; Flanagan, Lorna; McCawley, Niamh; Pabari, Ritesh; Kay, Elaine W.; McNamara, Deborah A.; et al. (2016): Targeting the 19S proteasomal subunit, Rpt4, for the treatment of colon cancer.. Royal College of Surgeons in Ireland. Journal contribution. <https://hdl.handle.net/10779/rcsi.10793306.v1>

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Targeting the 19S proteasomal subunit, Rpt4, for the treatment of colon cancer

Karen Boland^{1,2}, Lorna Flanagan¹, Niamh McCawley^{1,3}, Ritesh Pabari⁵, Elaine W. Kay⁴,
Deborah A. McNamara³, Frank Murray², Annette T. Byrne¹, Zebunnissa Ramtoola^{5*},
Caoimhín G. Concannon^{1*}, and Jochen H.M. Prehn¹

¹Centre for Systems Medicine and Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, 123 St Stephen's Green, Dublin 2, Ireland; Departments of ²Gastroenterology, ³Surgery, and ⁴Pathology, Beaumont Hospital, Beaumont, Dublin 9, Ireland; ⁵School of Pharmacy, Royal College of Surgeons in Ireland, York House, York Street, Dublin 2, Ireland

* Both authors contributed equally to this work.

To whom correspondence should be addressed:

Prof. Jochen H.M. Prehn
Department of Physiology and Medical Physics
Royal College of Surgeons in Ireland
123 St. Stephen's Green
Dublin 2, Ireland
Phone: +353 – 1 – 402 – 2255
FAX: + 353 – 1 – 402 – 2447
E-mail: jprehn@rcsi.ie

The authors disclose no potential conflicts of interest.

This research was supported by grants from the Health Research Board, Ireland (APOCOLON; TRA/2007/26; HRA_POR/2012/121) to JHMP, EWK and FM, and Science Foundation Ireland (13/IA/1881) to JHMP.

Word Count: 5,463

Abstract

Deregulation of the ubiquitin-proteasome pathway has been frequently observed in a number of malignancies. Using quantitative Western blotting of normal and matched tumour tissue, we here identified a significant increase in the 19S proteasome subunit Rpt4 in response to chemoradiation in locally advanced rectal cancer patients with unfavourable outcome. We therefore explored the potential of *Rpt4* reduction as a therapeutic strategy in colorectal cancer (CRC). Utilizing siRNA to down regulate Rpt4 expression, we show that silencing of *Rpt4* reduced proteasomal activity and induced endoplasmic reticulum stress. Gene silencing of *Rpt4* also inhibited cell proliferation, reduced clonogenic survival and induced apoptosis in HCT-116 colon cancer cells. We next developed a cell penetrating peptide-based nanoparticle delivery system to achieve *in vivo* gene silencing of Rpt4. Administration of Rpt4 siRNA nanoparticles reduced tumour growth and improved survival in a HCT-116 colon cancer xenograft tumour model *in vivo*. Collectively, our data suggest that inhibition of Rpt4 represents a novel strategy for the treatment of CRC.

Key words: proteasome, colorectal cancer, Rpt4, nanocomplexes, chemotherapy

1.0 Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related mortality in the United States according to data collected by the Centre for Disease Control (CDC) and the American Cancer Society, with an average lifetime risk of 5.1% (Jemal et al., 2010). Developments in adjuvant chemotherapy and radiotherapy have a role in increasing overall survival, although surgical resection remains the mainstay of curative treatment where clinically appropriate. However, resistance to (radio)chemotherapy is one of the leading causes of treatment failure in CRC (Violette et al., 2002). As such, novel therapeutic strategies are required to increase overall patient survival as well as providing alternative treatment paradigms to patients who become chemo-resistant to standard therapies.

Regulated degradation of proteins is central to the maintenance of cellular homeostasis. The majority of intracellular protein degradation is mediated via the ubiquitin-proteasome pathway (Ciechanover et al., 1991). The mammalian 26S proteasome is composed of a 20S core particle which houses the sites of active proteolysis within its inner β -subunit rings, and two 19S outer regulatory particles which are attached to the α -ring (Glickman and Ciechanover, 2002). This is a complex structure which influences many cellular processes including cell cycle, apoptosis, stress responses and cellular differentiation (Adams, 2004; Nakayama and Nakayama, 2006). Substrates of the ubiquitin-proteasome degradation pathway known to be regulators of these processes include the tumour suppressor p53, cyclins, I κ B α , Inhibitor of Apoptosis Proteins (IAPs), and Bcl-2 family of proteins (Jesenberger and Jentsch, 2002) (Zinszner et al., 1998). The 19S regulatory particles consist of a lid and base which act together to confer further substrate specificity to the process of proteasomal degradation. This 19S base has a ring of six AAA ATPases, Rpt1-6 (Regulatory Particle ATPases 1-6) (Braun et al., 1999). They enable ATP-dependent function of the 19S

regulatory particle with their role in the recognition of polyubiquitylated substrates for protein degradation, and substrate unfolding for translocation into the 20S core using reverse chaperoning (Adams, 2004; Braun et al., 1999). Therefore, the 19S subunits are essential for the degradation of these polyubiquitylated proteins which collectively form the largest group of proteasomal substrates.

Deregulation of the ubiquitin-proteasome pathway has been identified in a variety of malignancies (Bazzaro et al., 2006; Chen and Madura, 2005; Mani and Gelmann, 2005). The differential sensitivity of malignant cells to proteasome inhibition (Eldridge and O'Brien, 2009), leading to induction of cell death and apoptosis and enhanced sensitivity to chemotherapy have validated the use of proteasome inhibitors such as bortezomib for the treatment of multiple myeloma (Richardson et al., 2003) and mantle cell lymphoma (Fisher et al., 2006). Bortezomib is a reversible inhibitor of the 26S proteasome, and acts synergistically to enhance the effects of conventional chemotherapeutic agents and may overcome acquired resistance (Milano et al., 2007; Orłowski and Baldwin Jr, 2002). However, the use of bortezomib has been associated with dose related induction of neuropathies (Bruna et al., 2010; Cavaletti et al., 2007). Given the existence of potential resistance mechanisms and the unwanted side effects of broad proteasome inhibition, there is a need for more targeted approaches to proteasome inhibition. Increased activity of the proteasome has been noted in colon cancer (Arlt et al., 2009; Chen et al., 2009) and we have previously demonstrated that Rpt4 (PSMC6) is differentially expressed in colorectal cancer tissue when a panel of proteasomal subunits including 20S core, Rpt4 and polyubiquitylated proteins were tested. Analysis of Rpt4 protein levels using immunohistochemistry in Stage 2 node-negative patients with colon cancer showed that increased Rpt4 staining was associated with disease free survival as well as overall survival. This indicates that in Stage 2 colorectal

cancer that Rpt4 is a prognostic biomarker for these patients, and potentially select the patient cohort who may benefit from adjuvant chemotherapy (McCawley et al., 2012). Therefore, we tested the hypothesis that specific inhibition of the proteasome subunit Rpt4 (PMSC6) represents a novel strategy for the treatment of CRC.

2.0 Materials and Methods

2.1 Patient samples

All tissue was sourced from an institutional review board approved tissue bio-bank maintained at Beaumont Hospital, Ireland. Informed consent was obtained from each participating patient. A cohort of patients with colon cancer was selected from the Bowel Cancer database for which clinical follow up was available. Colonic resection specimens were sent fresh to the Pathology Department where a trained technician obtained tumour and adjacent normal tissue specimens, which were then snap frozen. Standard pathology reports for the resected colon tumours were issued. Biopsy tissue, both tumour and matched normal, was obtained from a separate cohort of rectal cancer patients at colonoscopy or rigid sigmoidoscopy, prior to neoadjuvant radio chemotherapy. Following treatment, surgical resection tissue was obtained for these patients. Histology was verified by an experienced pathologist, and tissues were evaluated to ensure consistent quality and tumour presence. Clinical follow-up and response to neoadjuvant radio chemotherapy was reported by a dedicated clinical research nurse. Specific ethical approval for this research was obtained from the Beaumont Hospital Research Ethics Committee in compliance with local protocols.

2.2 Western blotting

Patient samples were lysed in 400 ml ice-cold buffer -50 mmol/l HEPES (pH 7.5), 150 mmol/l NaCl, 5 mmol/l Na-EDTA and protease inhibitor (Sigma). Samples were kept on ice and homogenized using 30 s pulses with the Ultra-Turrax T25 Basic Homogeniser. Cell line-derived samples were lysed in SDS-lysis buffer [62.5 mM Tris/HCl (pH 6.8), Glycerin 10%, SDS 2%, H₂O]. Protein concentrations were determined using the standard Pierce Micro-BCA Protein Assay (Pierce, Northumberland, UK). Western blotting was performed using mouse monoclonal antibodies to Rpt4 (Enzo Life Sciences, Exeter, UK), Grp78/KDEL

(StressGen, Ann Arbor, MI, USA), pUbq (Biomol, Enzo Life Sciences, Exeter, UK), α -tubulin or β -actin (Sigma, Dublin, Ireland). Secondary antibodies coupled to horse radish peroxidase were detected using a Fujifilm LAS 4000 imager (Fuji, Sheffield, UK).

2.3 Cell culture and treatment

HCT-116 wt and p53 $-/-$ cells were obtained from Prof. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). The authenticity of the cell lines was confirmed by DNA STR profiling conducted by the DSMZ in October 2012 and all experiments were carried out within six months of authentication. Cells were grown in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-Glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. HT-29, LoVo and CRL 1807 cells were grown in DMEM containing 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were cultured at 37°C in 5% CO₂ and maintained in logarithmic growth by sub-culturing twice weekly. In indicated experiments cells were treated with a combination of 5-fluoururacil (30 μ g/ml) and oxaliplatin (10 μ g/ml) diluted in fresh medium or vehicle as control.

2.4 Transfection with siRNA

HCT116 cells were seeded at a density of 6×10^5 cells per well in a 24 well plate for 24 h prior to transfection using Metafectene (Biontex, Martinsried Germany) with siRNA duplexes (Sigma-Aldrich) with either a control sequence or Rpt4 targeting sequence at a final concentration of 50 nM of siRNA in 1 ml of OptiMEM. The sequences used were: UUCUCCGAACGUGUCACGUdTdT for control; GGAGUUAAGGGAACAAUUAdTdT for Rpt4 sequence 1; UAACUGUUUAAGCACUUCAdTdT for Rpt4 sequence 2.

2.5 Quantitative Polymerase Chain Reaction (qPCR) analysis

Cells were transfected with control siRNA or siRNA targeting Rpt4 (Sigma) when cells were 70% confluent for RNA extraction. RNA extraction was completed on ice using a Qiagen RNeasy Kit, following described protocol. Following RNA extraction, the RNA was frozen at -80°C until used. RNA was removed from the -80°C freezer and thawed once. RNA concentrations were measured using a Nanodrop, calculating concentration in ng/μl and generating stocks for a final concentration of 1 μg/μl with sterile RNA free H₂O. Sample volume was generated to eventually evaluate 10 samples per condition including two controls, one without Reverse Transcriptase. cDNA was then generated using Invitrogen reagents. 4 μl dN6 was added to 20 μl RNA/H₂O and cycled in thermal cycler for 5 min at 65°C. Temperature was then dropped to 4°C and again elevated to 25°C, and immediately the process was paused. Mastermix, generated using reagents from Invitrogen, (8 μl 5x buffer, 4 μl 0.1M DTT, 2 μl 10mM dTNP₃, 1 μl RNaseOUT and 1 μl SS11) was then added to the samples to make a total volume of 40 μl, with the exception of the Reverse Transcriptase-free control. The cycle was then resumed on the thermal cycler and when complete, frozen overnight at -20°C. qPCR analysis was performed using Lightcycler 2.0 (Roche Diagnostics). Primers tested included Actin, Rpt4 and Grp78. Samples were prepared using Invitrogen reagents. 3 samples per condition (control, Rpt4). We used two controls with either no reverse transcriptase or no template. 2 μl cDNA was added to appropriate light capillaries in a cooled box, followed by 2 μl RNase free H₂O. Mastermix was generated (SYBR Green [Qiagen], RNase free H₂O, 10 μM forward primer and 10 μM reverse primer) and a total volume of 18 μl was added to capillaries. After centrifugation at 3000 rpm for 15 seconds, these samples were analysed in the light cycler with 50 cycles and relative mononuclear quantification. The qPCR reactions were run initially at 95°C for 15 min following cycles of 94°C for 20 s, 59°C for 20 s and 72°C for 20 s.

Expression values were calculated having been normalized to Actin as a control target using the Lightcycler software and n-fold expression was then calculated using Microsoft Excel.

Primer sequences:

	<u>Sense Primer</u>	<u>Antisense Primer</u>
Actin	5'-GCTCCGGAAACTGGACATTA-3'	5'-GTCCCGTTTCGTCCTACAA-3'
Rpt4	5'-ACACTGGATCCTGCTTTGCT-3'	5'-TCGGAACATACCTGCTTCAG-3'
Grp 78	5'-TGCAGCAGGACATCAAGTTC-3'	5'-CTGCATGGGTGACCTTCTTT-3'

2.6 Clonogenic survival assay

At the appropriate time points post transfection cells were trypsinised, counted and 1000 cells were reseeded in a 60 mm tissue culture dish and incubated for 9 days in regular media. At day 9, the media was removed and the cell colonies were stained with 1 ml of Clonogenic Reagent (0.25% 1, 9-dimethyl-methylene blue in 50% ethanol) at room temperature for 45 min. Following incubation with the clonogenic reagent, plates were washed twice with PBS. Digital images of the plates were subsequently acquired and the number of colonies quantified and expressed relative to the control treated cultures.

2.7 Flow cytometry

Following treatment cells were then collected with trypsin- EDTA and washed once in PBS before resuspension in 100 µl of binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM Ca Cl₂) containing Annexin-V FITC conjugated (5 µl/ml) (BioVision, Mountain View, CA, USA) and Propidium Iodide (PI) (1 µg/ml). Cells were stained at room temperature for 20 min and then analysed immediately on a Partec Cyflow ML16 flow cytometer (Partec,

Münster, Germany) equipped with a 488 nm argon ion laser, a 532 nm diode laser and a 405 nm diode laser. Data was analysed using the Partec FloMax software.

2.8 Proteasome Activity Assay

Cells were plated at a density of 1×10^5 in a 12 well plate and incubated at 37° C for 24 h and then transfected as indicated. Cells were lysed after 72 h by addition of 500 µl of sterile filtered lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM Na-EDTA) to each well and plates were agitated for 3 min and then frozen at -80° C. In addition, wells of untransfected cells treated with 50 nM bortezomib for 3 h as a positive control were similarly lysed. For the activity assay 96 well black clear-bottomed plates were used, testing each condition in duplicate. Substrates were prepared on ice, adding 150 µl sterile filtered reaction buffer (25 mM HEPES pH 7.4, 1 mM EDTA, 0.1% CHAPS and 10% Sucrose) per well with 10 mM of appropriate substrate 3 (Sigma) measuring trypsin-like proteasome activity, 10 mM substrate 6 (Sigma) measuring chymotrypsin-like proteasome activity or 10 mM Caspase-like substrate (Z leu leu Glu-AMC, BML). 50 µl of each condition from 12-well plates were added to substrates. The fluorescence of each sample was measured at 380/460 nm using a microplate reader (GENios, Tecan) every min for 30 min. The Δ fluorescence/min was calculated by linear regression and was corrected for protein content.

2.9 MTT cell viability assay

Following treatment, thiazolyl blue tetrazolium bromide (MTT, 1 mg/ml; Sigma) was added to each well and incubated in the dark at 37°C. After 4 h, the medium was aspirated and the dark blue crystals were dissolved in DMSO (200 µl/well). The absorbance of each sample was measured at 560 nm using a microplate reader (GENios, Tecan). The percentage cell viability was calculated relative to the control treated cultures.

2.10 Subcutaneous mouse model of colon cancer

Bioware ULTRA cell lines HCT 116- luc2 were purchased from Caliper LS (MA, USA). These cells were treated *ex vivo* with either control siRNA or siRNA targeting Rpt4. After 48 h, 2.5×10^6 cells were implanted with subcutaneous injection into the right flank of Balb-c nu/nu mice (Charles River, USA). Ethical approval was granted by our local and institutional ethical committee. The mice were imaged 24 h post implantation to ensure cell viability using an IVIS Spectrum scanner (Caliper LS). Imaging occurred 10 min post intraperitoneal administration of 150 mg/kg body weight D-luciferin (Caliper LS). Weekly bioluminescent imaging occurred thereafter, until endpoint (tumour size) 15 mm. Tumour size was measured and mouse weights were recorded every 72 h. Tumour volume was then calculated using a previously published modified ellipsoidal formula (Euhus et al., 1986). After killing, the tumour and internal viscera were analysed and imaged *ex vivo*.

2.11 Formulation and characterisation of CPP:siRNA nanoparticles

The cell penetrating peptide, Octa-arginine (CPP) (Genscript, NJ, USA) was dissolved in RNA-ase free water to achieve required concentrations. siRNA of either the control sequence or Rpt4 was added dropwise to CPP solution, at ratios of 9:1, 25:1 and 50 : 1 CPP : siRNA. After addition of the siRNA, the solution was stirred on magnetic stirrer for 6 h at room temperature to form nanoparticles. The average particle size, polydispersity index (PDI) and zeta potential (ZP) of the nanoparticles was measured using a Zetasizer (Nanoseries, Nano-ZS, Malvern Instruments,UK). The data is presented as the average of 5 measurements +/- standard deviation. Efficacy of siRNA; CPP nanocomplexes were assessed by transfection of HCT-116 WT luc-2 ULTRA cells seeded on a 96 well plate, drawing comparison with transfection of siRNA using metafectene and using CPP only as a negative control. MTT

assay enabled analysis of cytotoxicity in response to Rpt4 siRNA:CPP complexes. 4 solutions for intratumoural delivery were prepared, at a ratio of 50:1 CPP:siRNA. siRNA duplex (Sigma) concentrations were added dropwise to these solutions at concentrations of 1 mg/kg Rpt4 siRNA, 0.3 mg/kg Rpt4 siRNA, 0.1 mg/kg siRNA integrated into R8 nanocomplexes, and CPP alone as a control.

2.12 *In vivo* delivery of siRNA nanocomplexes

CPP:siRNA nanoparticles were delivered *in vivo* to mice with established subcutaneous tumours at least 3 x 3 mm in size. Using nanoparticles prepared on the day of use, 6 intratumoural injections were delivered to each mouse at an interval of 72 h apart with serial measurement of tumour size using calipers. Prior to injection, the mouse was anaesthetised with general inhalational anaesthetic and the overlying skin was sterilized. A volume not exceeding 50 µl of CPP:siRNA solution or CPP alone was injected. Puncture sites were alternated to minimize trauma induced local inflammatory reactions and ulceration.

3.0 Results

3.1 Rpt4 protein levels increase in rectal tumours not responding to chemoradiation therapy

Locally-advanced rectal cancer patients frequently receive neoadjuvant chemoradiation therapy prior to surgery in an attempt to downstage the tumour so it can be more completely removed at surgery. Approximately 40% of patients show no response to this treatment and display no tumour regression (Janjan et al., 2001; Lu et al., 2013). To explore the role of Rpt4 in response to neoadjuvant chemoradiation, we obtained quality-assured rectal cancer biopsies prior to chemoradiation therapy. Western blot and subsequent densitometry analysis of pre-treatment tumour biopsy and matched normal tissue from 24 rectal cancer patients suggested that Rpt4 protein levels did not correlate with clinical responses as assessed by RCPATH staging in both normal and tumour tissue (Fig. 1A).

For those patients who did not respond to neoadjuvant chemoradiation therapy we also obtained post treatment surgical resection tissue, obtained following neoadjuvant chemoradiation therapy (n=6). This allowed us to compare Rpt4 protein expression pre- and post-treatment to assess any acute changes in protein expression in response to treatment in poor responding patients (Fig. 1B). Of note, neoadjuvant chemoradiation therapy significantly increased protein expression levels of Rpt4 in poor responding patients (Fig. 1B,C, $P = 0.039$), suggesting that an increase in Rpt4 protein levels may be associated with resistance to chemoradiation therapy. Increased Rpt4 protein expression was not seen in normal tissue (Fig. 1D), suggesting that this increase was limited to tumour tissue.

To investigate the association of Rpt4 protein levels with long term patient outcome we also examined Rpt4 protein levels in the resected primary tumour of a subset of 26 stage 2 and 3 CRC patients. Kaplan Meier survival analysis of tumour tissue revealed that patients with Rpt4 levels below mean levels exhibited a statistically significant increase in survival time when compared to patients with above mean levels of Rpt4 protein (Fig. 1E, P = 0.032). This correlation between increased survival and above mean levels of Rpt4 protein was not seen in patient matched normal mucosa (Fig. 1F). This indicates that increased Rpt4 protein levels of normal tissue is not a prognostic marker for Stage 2 and 3 CRC patients in the setting of adjuvant treatment.

3.2 Increased expression of Rpt4 in colon cancer cell lines

We next investigated whether colon cancer cell lines exhibited detectable levels of Rpt4 protein. In a panel of colon cancer cells with differing *K-ras* and *TP53* status (HCT 116 *wt* and *p53*^{-/-}; Colo 205, DLD-1, HT-29, and LoVo) the expression levels of Rpt4 were analysed by Western blotting and densitometric analysis (Fig. 2A). In addition, we included samples derived from a non-transformed colon epithelial cell line, CRL 1807 (ATCC). As demonstrated in Fig. 2A,B, all of the colon cancer cell lines displayed similar levels of Rpt4 expression. The non-transformed CRL 1807 had lower levels of Rpt4 protein when compared to the tumour cell lines. However, analysis of 20S core expression in these colon cancer cell lines and CRL 1807 did not mirror this increased proteasome subunit expression in the cancer cell lines (Fig. 2C,D)

3.3 Small interfering RNA (siRNA) mediated knockdown of *Rpt4* reduces proteasomal activity

To explore whether Rpt4 inhibition is a feasible approach for the treatment of colon cancer, we next sought to investigate the effects of inhibition of *Rpt4* expression. We could demonstrate efficient knockdown of *Rpt4* expression using two different siRNA sequences, with sequence 2 showing a more pronounced inhibition of *Rpt4* expression (Fig. 3A). *Rpt4* siRNA sequence 2 was therefore depicted for all of the subsequent experiments. We next investigated the potential of *Rpt4* gene silencing on the function of the catalytic activity of the proteasome. We assayed for the three different enzymatic activities associated with the 26S proteasome. Chymotrypsin-like activity of the proteasome is thought to be most representative of whole proteasomal function (Kisselev et al., 2006; Kisselev and Goldberg, 2005). As demonstrated in Fig. 3B gene silencing of *Rpt4* significantly reduced chymotrypsin-like proteasomal activity, but inhibition of trypsin-like activity was not statistically significant. This was mirrored by the less marked reduction in trypsin-like activity after treatment with bortezomib (Fig. 3C). In contrast, the 26S subunit proteasome inhibitor bortezomib reduced both chymotrypsin- and trypsin-like protease activity. Caspase-like activity was also affected by *Rpt4* silencing (Fig. 3D).

3.4 Gene silencing of *Rpt4* induces endoplasmic reticulum (ER) stress

As a component of the 19S proteasome, Rpt4 is not directly involved in the proteolytic activity associated with the proteasome, but in the unfolding of the proteasome substrates and their movement into the 20S catalytic core of the proteasome for degradation. We therefore also explored whether *Rpt4* gene silencing induced markers of the unfolded protein response by inhibiting the degradation of unfolded proteins targeted for the proteasome. Indeed, transfection of HCT-116 cells with *Rpt4* siRNA induced a significant upregulation of *grp78* mRNA, a marker of endoplasmic reticulum stress and a target gene of the unfolded protein response (Fig. 4A). This was also confirmed on the protein level, with increased

grp78 protein and increased accumulation of poly-ubiquitinated proteins evident in HCT116 cells after *Rpt4* gene silencing (Fig. 4B,C).

3.5 *Rpt4* deficiency induces apoptosis and reduces clonogenic survival in HCT-116 colon cancer cells

Next we investigated the functional consequences of decreased Rpt4 expression on the survival and growth of colon cancer cells *in vitro*. In order to quantitatively analyse the effects of Rpt4 knockdown on cell survival and proliferation we performed flow cytometry analysis to measure phosphatidylserine exposure using Annexin V staining as a marker of apoptotic cell death. One of the major transcription factors whose expression is regulated by ubiquitylation and subsequent degradation by the proteasome is p53 which is frequently mutated in cancer cells. Previous studies have demonstrated that proteasomal inhibition induced cell death is at least in part p53 dependent (Concannon et al., 2006). Therefore, we also included *p53*-deficient HCT-116 cells in these studies. Treatment of HCT-116 colon cancer cells or *p53*-deficient HCT-116 colon cancer cells with *Rpt4* siRNA resulted in a significant time dependent increase in the number of cells staining positive for Annexin V, suggesting that *Rpt4* reduction was sufficient to increase the levels of spontaneous cell death. The effect on cell survival was more pronounced in wild-type HCT-116 cells, with approx. 40% of cells undergoing apoptosis after 72h (Fig. 5A). When the non-transformed colon epithelial cell line, CRL 1807, was treated with *Rpt4* siRNA, there was no increase in cell death levels when compared to control-transfected CRL 1807 cells (Fig. 5B) despite *Rpt4* reduction in these cells (Fig. 5C).

To further assess the effects of *Rpt4* inhibition, clonogenic survival assays were performed to examine survival and proliferation of HCT-116 cells following inhibition of *Rpt4* expression.

HCT-116 cells were transfected with either control siRNA or siRNA sequences targeting *Rpt4*. Following siRNA mediated knockdown for 72 h, the cells were counted and 1000 cells plated onto a 6 cm culture dish where they were allowed to expand for 10 days (Fig. 5D). The number of colonies formed in cells transfected with *Rpt4* siRNA was significantly reduced compared to cells transfected with the control siRNA sequence. Of note, silencing of *Rpt4* also induced strong effects in HCT-116 *p53*^{-/-} cells that were comparable to those seen in HCT-116 cells. Again, when the non-transformed colon epithelial cell line, CRL 1807, was treated with *Rpt4* siRNA, there was no significant reduction in clonogenic survival when compared with control-transfected CRL 1807 cells (Fig. 5E).

3.6 *In vivo* *Rpt4* gene silencing using nanocomplexes as a potential strategy for the treatment of CRC

Cellular delivery of siRNA is challenging due to its polyanionic nature, its rapid degradation and short *in vivo* half-life. We finally investigated a strategy for the delivery of *Rpt4* siRNA *in vivo*, using nanoparticles formed by electrostatic complexation of a cell-penetrating peptide, Octa-arginine (CPP). Characterization of these nanocomplexes demonstrated that particle size of 237.9 nm was achieved with increasing ratios of CPP. The charge of the siRNA was also neutralized and a net overall positive charge was achieved with increasing CPP ratio (Table 1). In order to evaluate the optimal means to achieve *Rpt4* gene silencing *in vitro* and *in vivo*, CPP nanocomplexes generated with a variety of CPP:siRNA ratios were added to HCT-116 cells cultured *in vitro*. Cell death in response to transfection of siRNA using these nanocomplexes was compared to our established protocol for transfection of siRNA using Metafectene as a positive control. Cell death in response to *Rpt4* gene inhibition using these CPP:siRNA nano-complexes at a ratio of 50:1 was sustained at 12 h and 48 h post

transfection at a level exceeding that of metafectene based transfection protocols (Fig. 6 A, B).

Next, using a HCT-116 luc2 ULTRA colon cancer tumour mouse model, nanoparticles were delivered via local intratumoural injection, increasing the likelihood of intracellular release of the siRNA payload and hence achieving the therapeutic goal of *Rpt4* gene silencing. We found that mice with subcutaneous colon cancer cell line derived tumours had smaller tumour burden, with consequent enhanced survival benefit at all doses of nucleic acid targeting *Rpt4* (Fig. 6C, D). Interestingly, at doses of 0.1 mg/kg siRNA delivered over 9 doses, optimal survival advantage was achieved (average survival 45.7 days) compared with tumour cells transfected with control siRNA (average survival 33.5 days) in a statistically significant manner (Log Rank Mantel Cox Test) (Fig. 6E, F).

4.0 Discussion

Due to the significance of the ubiquitin-proteasome pathway (UPP) in cell signalling, proteasome inhibitors have emerged as a new group of chemotherapeutic agents for the treatment of malignancies. Although the effects of proteasome inhibition on the activation of signaling pathways and downstream processes may vary in different cancers (Bajorek et al., 2003), these agents have demonstrable therapeutic benefits in both haematological (Arnold and Grune, 2002) and solid malignancies (Crawford et al., 2011; Johnson, 2015; Kisselev and Goldberg, 2001), either as sole therapeutic agents or in combination with cytotoxic therapies (Bazzaro et al., 2006; Cusack, 2003; Russo, 2001). Proteasome inhibitors have been shown to inhibit cell-cycle progression and to induce apoptosis and cell death in a wide range of cancer cells *in vivo* and *in vitro* (Crawford et al., 2011; Ling et al., 2003). They act through a variety of mechanisms, including p53 stabilization, alterations in gene expression, and inhibition of

caspase degradation, among many others (Eldridge and O'Brien, 2009). Proteasome inhibitors have also been shown to overcome chemotherapy and radiotherapy resistance through inhibition of I κ B degradation (Chen et al., 2011; Reddy and Czuczman, 2010; Russo, 2001). However, targeting the ubiquitin-proteasome system using a non-specific proteasome inhibitor such as bortezomib in malignancy is limited by the wide range of side effects to which patients may be subjected given that over 80% of intracellular proteolysis is controlled and effected by the proteasome (Wang and Maldonado, 2006). As a result, proteasome inhibitors have a narrow therapeutic index. Bortezomib targets the 20S proteasome catalytic core, attenuating cell proliferation and survival, and has proven effect in metastatic colorectal cancer (Kozuch et al., 2008). Other mechanisms seen in cancer cells treated with this drug include reports of upregulation of pro-survival proteins in response to proteasome inhibition (Milano et al., 2009), and stability of p53 expression in treated cells and tissues, although pro-apoptotic effects are also demonstrated in cells which do not express p53 (Lenz; Ling et al., 2003). In addition, the property of differential susceptibility of cancer cells to bortezomib and other proteasome inhibitors is part mediated by NF-kappaB pathways (Hideshima et al., 2009). Alternative therapeutic approaches may target components of the 19S proteasomal subunit, modulating proteasome assembly and the efficacy of individual proteasome subunits. Diversification of therapeutic agents within the Ubiquitin-Proteasome Pathway spectrum may offer alternatives to bortezomib with improved side-effect profiles and still harness the potential for synergistic enhancement of chemotherapy effect when used in tandem with proteasome inhibition. These factors have encouraged efforts to seek novel and more specific therapeutic targets for cancer therapy within the ubiquitin-proteasome system. We here tested the hypothesis that Rpt4, as a 19S proteasome subunit may be a valid target for the treatment of CRC.

We focused on the role of Rpt4 in colon cancer cell survival, as Rpt4 is a component of the 19S proteasome and not directly involved in the proteolytic activity associated with the proteasome. Rather, Rpt4 is involved in the unfolding of the proteasome substrates and their movement into the catalytic core of the proteasome for degradation. We have demonstrated that Rpt4 inhibition inhibited cell proliferation, reduced clonogenic survival, and induced apoptosis in colon cancer cells, and that this was associated with an increased ER stress response. p53 wt cells had higher levels of apoptosis on flow cytometry than p53 *-/-* cells. However there was no significant difference in attenuation of clonogenic survival after Rpt4 reduction in p53 *-/-* and p53 wt cells. p53 induction has previously been shown after use of proteasome inhibitors in cancer cells (Lopes et al., 1997), and proteasome inhibitor-induced apoptosis has been shown to be largely p53-dependent (Concannon et al., 2006). In contrast, clonogenic survival assays are a read-out of multiple cellular processes where apoptosis is not the sole determinant (Lo Nigro et al., 2007; Russell and Ling; Russell et al., 1995). Our data suggest that there are multiple, p53-independent anti-proliferative mechanisms involved in the response to *Rpt4* silencing, such as transcriptional machinery assembly (see below).

Rpt4 is a member of the AAA class of ATPases, forming a heterohexameric ring with Rpt 1-6, which attaches to the base of the 19S regulatory particle. Finley and colleagues have shown in yeast studies that the C termini of Rpt4 and Rpt6 are crucial for assembly of this regulatory particle (Park et al., 2011; Park et al., 2009). The effects of destabilisation of the regulatory particle and core particle interactions are demonstrated in our assays of reduced proteasome activity in response to *Rpt4* gene silencing. This reduction in activity may also relate to lower RP concentrations in the cell resulting in less 26S proteasome formation. We have found that Rpt4 inhibits proteasome activity, with significant inhibition of chymotrypsin-like and caspase-like activity (Fig. 3). Although there was a trend towards

inhibition of trypsin-like activity, this did not reach statistical significance. However, our chosen proteasome activity assay may overestimate the effect of Rpt4 on this activity as Rpt4 inhibition is likely to lead to lower cellular RP concentrations which would lead to CP presentation in a closed conformation and hence reduce potential fluorescent peptide cleavage.

It should also be noted that while Rpt 1-6 have critical roles in the unfolding of polyubiquitinated substrates for translocation into the proteasome core and subsequent proteolysis, within the regulatory particle they do not have identical functions. While their presence is required for the stoichiometric stability of the proteasome complex, individual mutations affect proteasome activity and non-proteolytic functions in a variety of manners, outlining their functional heterogeneity (Bar-Nun and Glickman, 2012; Rubin et al., 1998). Tumours bear hallmarks of genomic instability which has been exploited to unveil potential novel therapeutic targets and mechanisms of pathogenesis. The CYCLOPS genes identified a panel of genes which demonstrate partial loss in tumours, rendering them dependent of remaining alleles. Many of these genes are involved in proteasome activity such as PSMC7 (Rpt1). Inhibition of the remaining allele expressing PSMC7 led to attenuated tumour growth and inhibition of cell proliferation (Nijhawan et al., 2012). Furthermore, 26S proteasome levels may be limited by 19S complex levels as there was more expression of 20S, and we believe that inhibition of these 19S subunits including Rpt4 which has a strong assembly phenotype, induces proteasome stoichiometric instability and reinforces the argument to target these subunits to inhibit tumour development and growth.

Interestingly, proteasomal subunits can also perform functions independent of the proteolytic activity of the proteasome. 19S subunits have been reported to play a role in transcriptional

machinery assembly (Truax et al., 2010). Indeed, recent evidence points to the ability of 19S subunits to regulate transcriptional events. In particular, analysis of proteasomal independent functions of ATPases including Rpt4 and Rpt6 in *Saccharomyces cerevisiae* has demonstrated that these have alternative roles in gene silencing and achieve this by linking chromatin modifications (Ezhkova and Tansey, 2004). In HCT-116 colon cancer cells, an AAA ATPase of the 19S subunit was identified as a TNF-receptor associated protein-1 (TRAP-1) interacting protein, with effects on ubiquitylation independent of proteasomal function (Amoroso et al., 2011). While there is an established link between histone ubiquitylation and transcription contributing to these non-proteolytic functions of Rpt4, these pathways and mechanisms have not been fully uncovered. In addition, *Rpt4* gene mutations in yeast are associated with a phenotype particularly vulnerable to ER stress and with participation in aspects of ERAD. These characteristics are not mirrored by other ATPase mutations (Lipson et al., 2008). Further investigations will be required to elucidate the factors involved in the beneficial effect of Rpt4 inhibition on tumour cell proliferation.

The pronounced effects on cell death and attenuation of clonogenic survival noted *in vitro* as well as the increased expression of Rpt4 in non-responding rectal tumours as seen in Fig. 1, drove our hypothesis that *Rpt4* gene silencing may reduce tumour progression *in vivo*. Ideally we would also like to compare expression of Rpt4 in rectal tissue of a non-diseased cohort. In our subcutaneous tumour model in Balb/c nu-nu immunodeficient mice using luciferase expressing HCT 116 cells, cells transfected with siRNA targeting *Rpt4 in vivo* generated smaller tumours and a statistically significant survival improvement over control groups. Our *in vivo* methods were therefore proof of principle studies to address our hypothesis that *Rpt4* may attenuate tumor growth in an animal model. Further data in the area of adverse effects of this more specific proteasome target would allow further

comparison with non-specific proteasome inhibitors in terms of unwanted systemic symptoms and consequences.

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Figure Legends

Figure 1: Neoadjuvant chemoradiation therapy significantly increased protein levels of Rpt4 in poor responding patients. Biopsy tissue samples of 24 rectal cancer patients were obtained at colonoscopy or rigid sigmoidoscopy. **(A)** Densitometric analysis of Rpt4 expression levels relative to β -actin in biopsy tissue, tumour (T) and matched normal (N), of patients with varying pathological responses to radiochemotherapy. **(B)** Representative Western blot images of expression levels of Rpt4 (44 kDa) in tumour (T) and matched normal tissue (N) from both biopsy and surgical resection tissue. Probing with β -actin (42 kDa) was applied as a loading control. **(C)** Densitometric analysis of Rpt4 expression levels relative to β -actin in biopsy tissue and matched surgical resection tissue indicates that neoadjuvant chemoradiation therapy significantly increased expression levels of Rpt4 (n=6; *P = 0.039, log-rank test). **(D)** Densitometric analysis of Rpt4 expression relative to β -actin tumour and matched normal tissue. Rpt4 expression is not significantly increased in normal mucosa, suggesting that this is specific to tumour tissue (n = 6; P > 0.05, log-rank test) **(E)** Kaplan Meier survival analysis of high and low levels of Rpt4 relative to β -actin in tumour tissue stage 2 and 3 colon cancer patients. Survival was prolonged in the patient group that had below mean levels of Rpt4 [*P = 0.032, n = 26, log-rank (Mantel Cox) test]. **(F)** Kaplan Meier survival analysis of high and low levels of Rpt4 relative to β -actin in matched normal tissue of stage 2 and 3 colon cancer patients. [P = 0.48, n = 26, log-rank (Mantel Cox) test].

Figure 2: Rpt4 and 20S proteasome subunit levels in colon cancer cell lines. (A)

Representative cropped Western Blot analysis of Rpt4 (44 kDa) expression levels in a panel of colon cancer cell lines and the epithelial cell line CRL 1807. Probing with α -tubulin (50 kDa) was applied as a loading control. **(B)** Densitometric analysis of Rpt4 expression levels

in colon cancer cell lines expressed relative to the non transformed colon epithelial cell CRL 1807. **(C)** Western Blot analysis of 20S core particle (BioMol) expression levels in colon cancer cell lines with α -tubulin applied as loading control. **(D)**: Densitometric analysis of 20S core particle expression levels in colon cancer cells. Expression of Rpt4 and 20S was normalised to α tubulin and then compared to CRL 1807. Data are mean +/- S.E.M of n=3 independent determinations.

Figure 3: *Rpt4* gene silencing modulates proteasomal activity in a HCT-116 colon cancer cells. **(A)** Western Blot of Rpt4 expression in HCT 116 WT cell lines following transfection with two different siRNA sequences targeting Rpt4 in comparison with a control siRNA sequence. Samples were harvested 48 h after transfection. Probing with α -tubulin served as a loading control. **(B)** Inhibition of Rpt4 expression was confirmed using qPCR (*P = 0.0025). Data represent mean +/- S.E.M. (*P < 0.05, unpaired t-test, n=3.) **(C-E)** HCT 116 *wt* cells were treated for 72 h with either scrambled siRNA or a siRNA targeting Rpt4. Cells were lysed and agitated prior to analysis of proteasome activity. Proteasome activity was analysed using analysis of fluorescence levels following addition of reaction buffer and substrate specific to **(C)** chymotrypsin-like, **(D)** trypsin-like or **(E)** caspase-like. A fourth group of untransfected cells was treated 4 hs prior to analysis with the proteasome inhibitor Bortezomib (Bort.; 50 nM) as indicated. Activity was assessed as described in the materials and methods. Data are mean +/- S.E.M of n=4 independent samples per group in **C, D** and n = 3 in **E**. *P < 0.05 compared to Control siRNA group (ANOVA post-hoc Tukey).

Figure 4 : *Rpt4* gene silencing induces proteasomal and ER stress. **A:** Using qPCR analysis, we evaluated expression levels of markers of ER stress in HCT 116 WT colorectal cancer cells, 24 hs after treatment with siRNA targeting Rpt4 (Sigma). Upregulation of

expression levels of Grp78 (*P = 0.0189) was observed, with expression over 2 fold higher in cells following treatment with siRNA targeting Rpt4. This is in keeping with our analysis that ER stress as well as induction of the UPR in response to ERAD impairment occurs in this colorectal cancer cell line following Rpt4 knockdown. Inhibition of Rpt4 expression was confirmed using qPCR (*P = 0.0025). Data represent mean +/- S.E.M. (*P < 0.05, ANOVA post-hoc Tukey, n = 3.) **B:** 24 h following treatment of HCT 116 WT colon cancer cells with siRNA targeting control sequence or Rpt4, Western blot analysis was used to evaluate expression of polyubiquitylated proteins and Grp 78/KDEL, markers of proteasomal stress and ER stress respectively. α -tubulin was used as a loading control. **C:** The expression of polyubiquitylated proteins and Grp 78/KDEL was analysed by densitometry in Rpt4 knockdown cells relative to control. Data represent mean +/- S.E.M (n = 3 independent determinations).

Figure 5: Inhibition of Rpt4 expression induces cell death and attenuates clonogenic survival of colon cancer cells. (A) HCT116 cells and (B) CRL 1807 cells were transfected for 72 h with siRNA targeting Rpt4 or control siRNA and cell death was measured by Annexin V staining and subsequent flow cytometry. *P < 0.05 compares to Control siRNA (ANOVA post-hoc Tukey). (C) Western Blot analysis of Rpt4 expression levels CRL 1807 with α -tubulin applied as loading control. CRL 1807 non transformed colonic epithelial cells were transfected using siRNA targeting Rpt4 (Sigma) to achieve *Rpt4* gene silencing, and Rpt4 expression was assessed after 48 h. (D) HCT116 wt, HCT116 p53^{-/-} cells and (E) CRL 1807 non transformed colon epithelial cells were transfected with either Control or Rpt4 siRNA (Sigma) for 72 h and clonogenic survival assays performed. Colonies were counted and expressed relative to the control siRNA transfected cells of each genotype. Data are mean

+/- S.E.M from n=3 independent determinations per time condition. *P < 0.05 compared to Control siRNA treated cells (ANOVA post-hoc Tukey).

Figure 6: Nanocomplex-mediated delivery of small interfering RNA targeting the 19S proteasomal subunit, Rpt4, reduces tumour growth and increases survival *in vivo*. (A, B) Characterization of the effect of nanocomplexes on cell survival *in vitro*. In order to evaluate the optimal means to achieve *Rpt4* gene silencing, CPP:siRNA complexes were used at a ratio of 50:1. Cell death of HCT116 cells in response to *Rpt4* gene inhibition using the CPP:siRNA complexes was sustained at (A) 12 h and (B) 48 h post transfection at a level exceeding that of metafectene based transfection protocols. Cell death was evaluated using an MTT assay. Data represent mean +/- S.E.M. (*P < 0.05, unpaired student t-test, n = 6). (C) Tumour growth *in vivo*. Photographic representation of tumour burden in mice implanted with HCT 116 WT-luc2 ULTRA colon cancer cells (Caliper LS) and treated *in vivo* using intratumoural injection of control scrambled sequence siRNA or siRNA targeting Rpt4 at doses of 0.1 mg/kg recorded serially before and during treatment. (D) Tumour Bioluminescence *in vivo*. Photographic depiction of tumour bioluminescence in p/sec/cm² recorded serially in mice representative of study groups shown. (E,F) Kaplan Meier survival analysis of tumour bearing mice treated *in vivo* using intra-tumoural injection of control scrambled sequence siRNA (n = 6) or siRNA targeting Rpt4 at doses of 0.1 mg/kg, 0.3 mg/kg and 1 mg/kg (n = 8 per group). Control groups are identical in Figure 5 D and E, but graphs are shown separately for clarity. Survival was prolonged in all groups treated with siRNA targeting Rpt4 but was most marked in the group treated at a dose of 0.1 mg/kg. Statistical significance was reached in the groups treated at doses of 0.1 mg/kg and 1.0 mg/kg. Data represent mean +/- S.E.M. [*P < 0.05, log rank (Mantel Cox)].

Figure 1

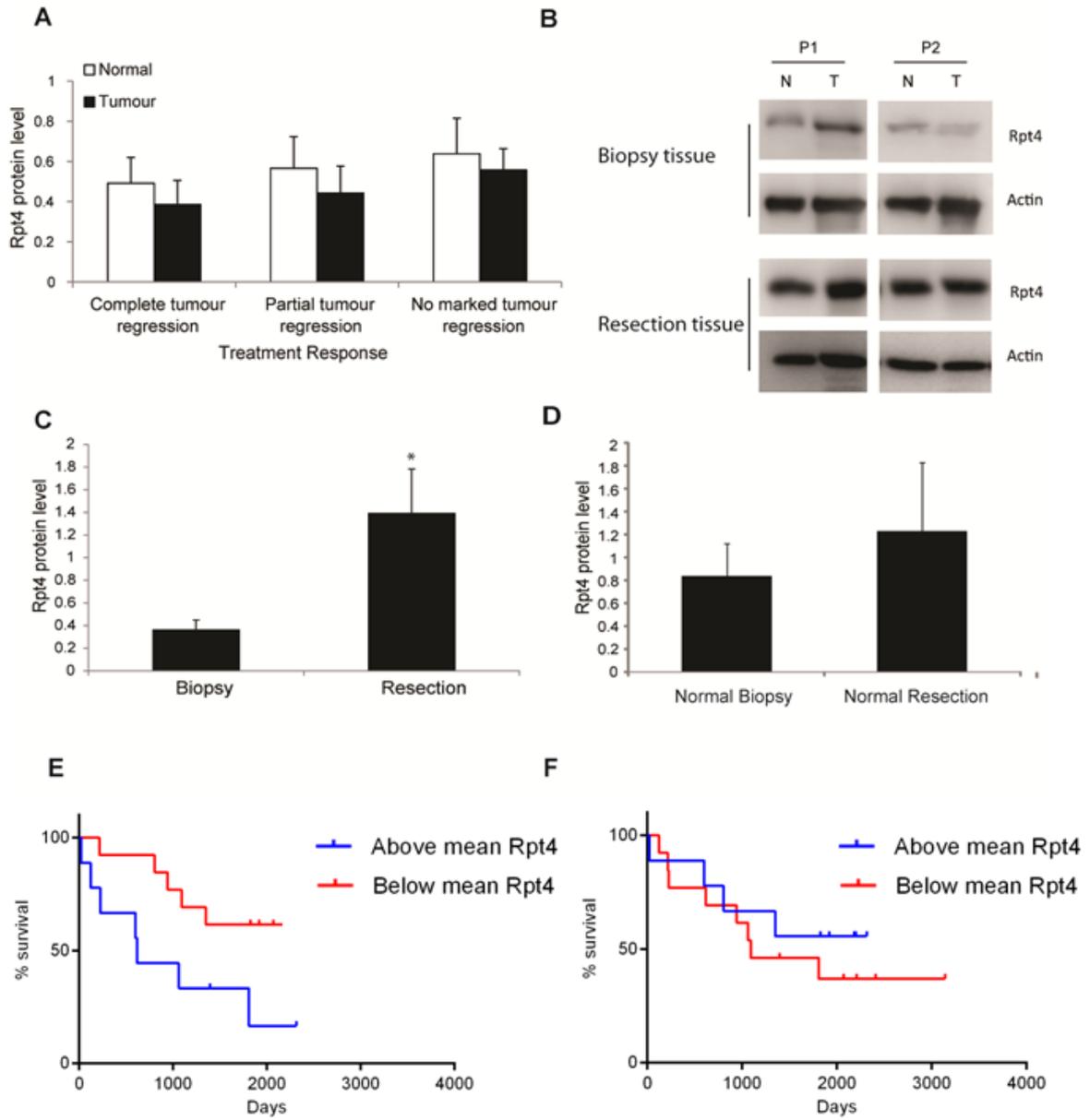


Figure 2

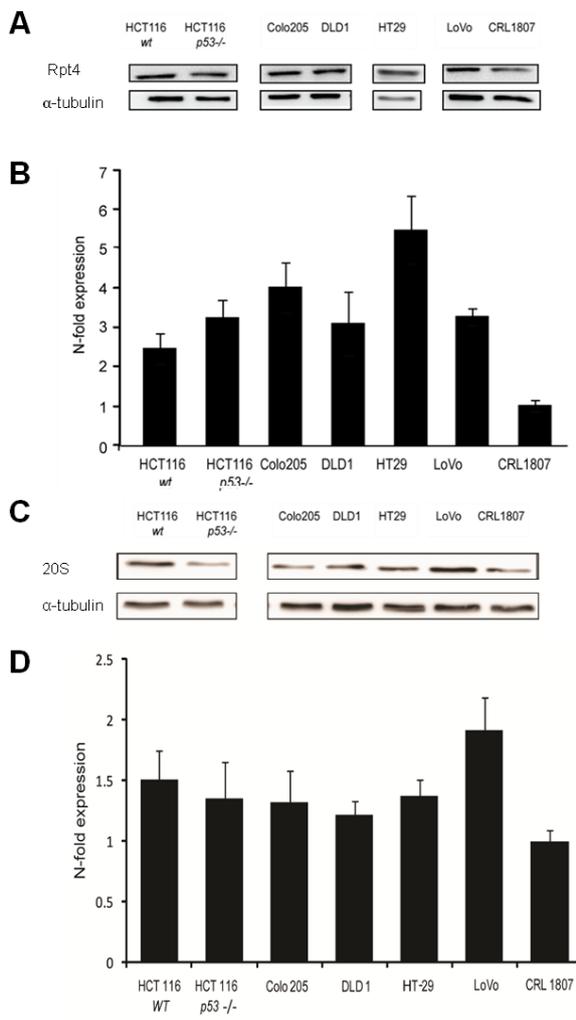


Figure 3

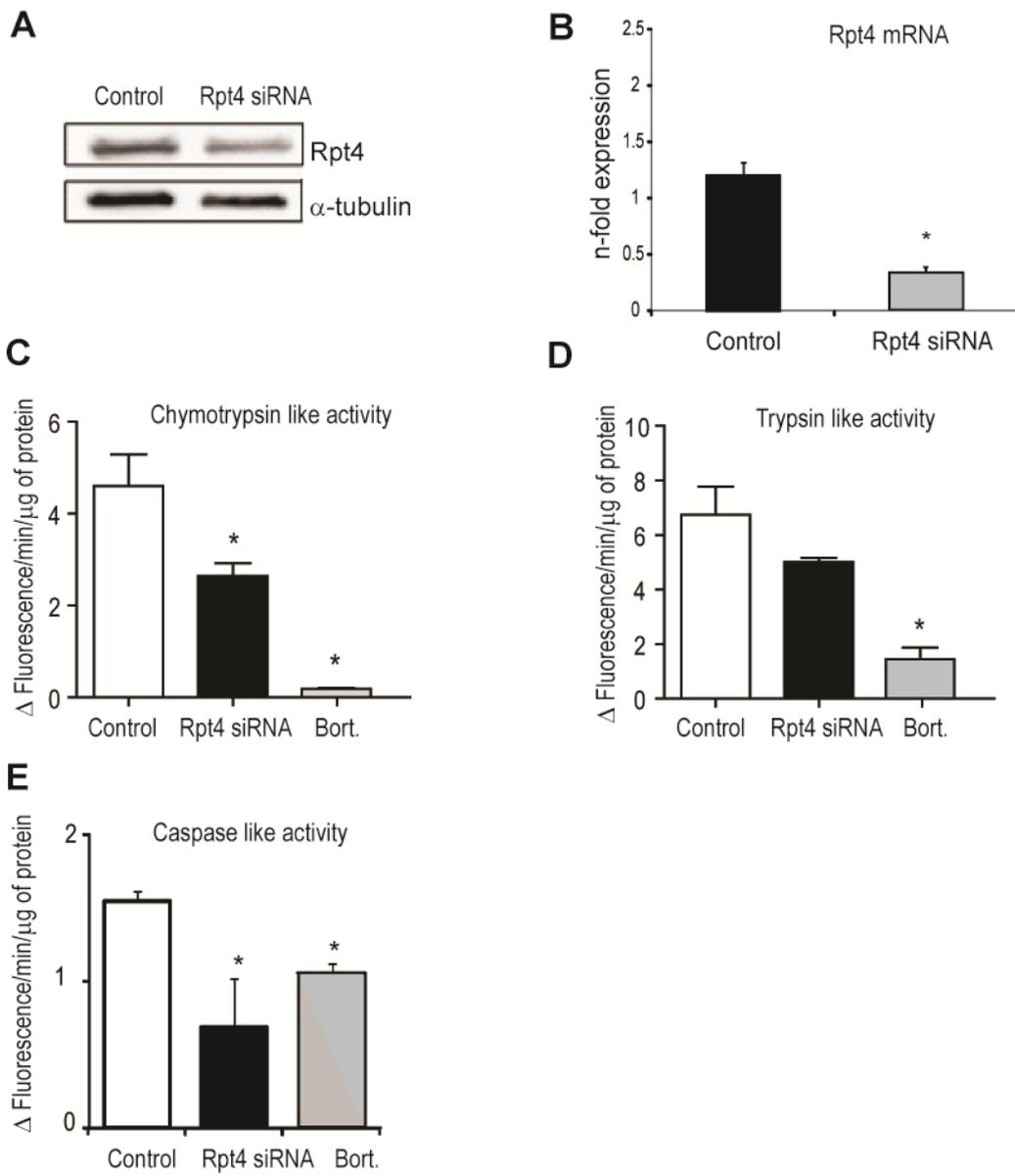


Figure 4

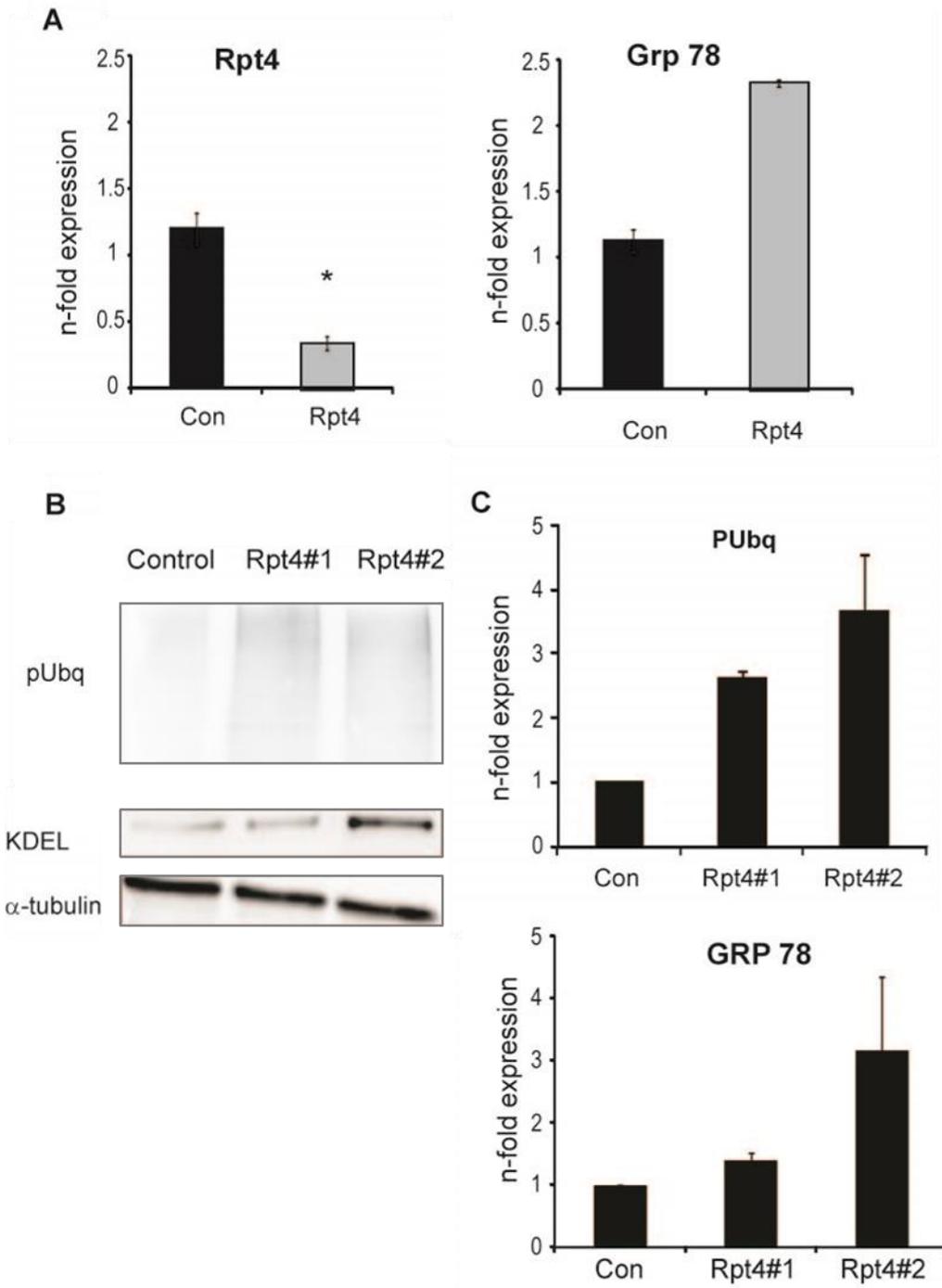


Figure 5

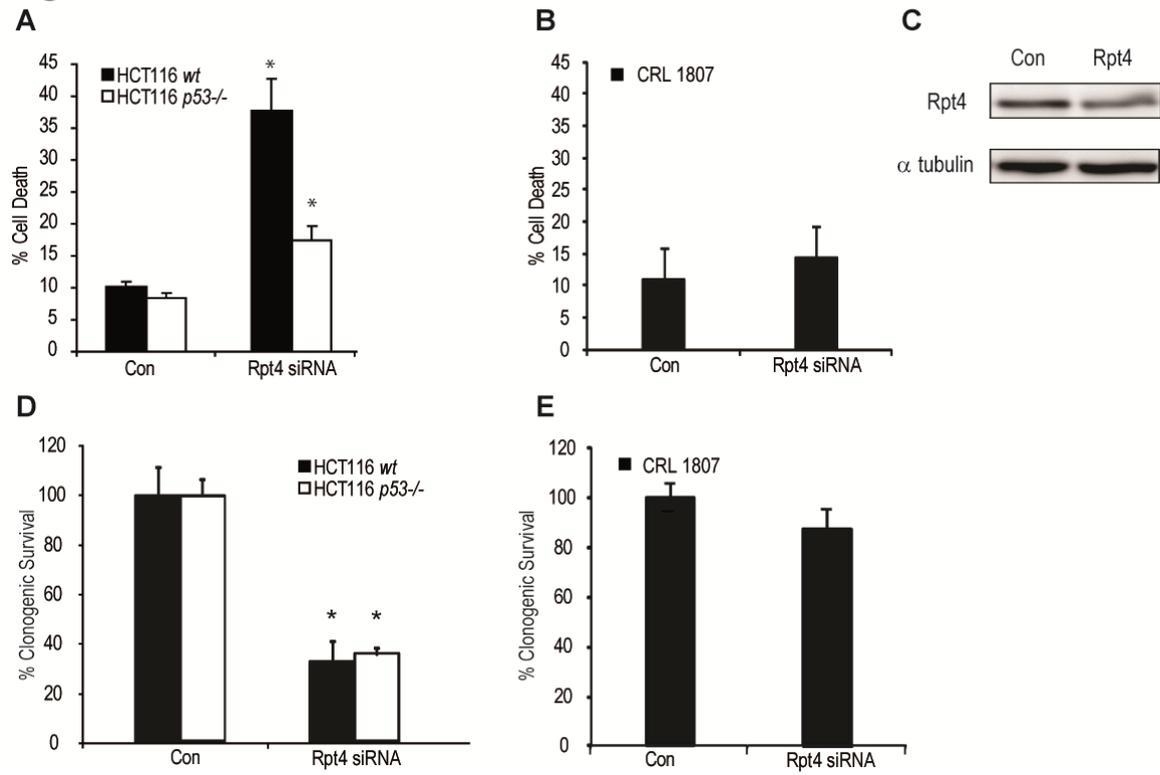


Figure 6

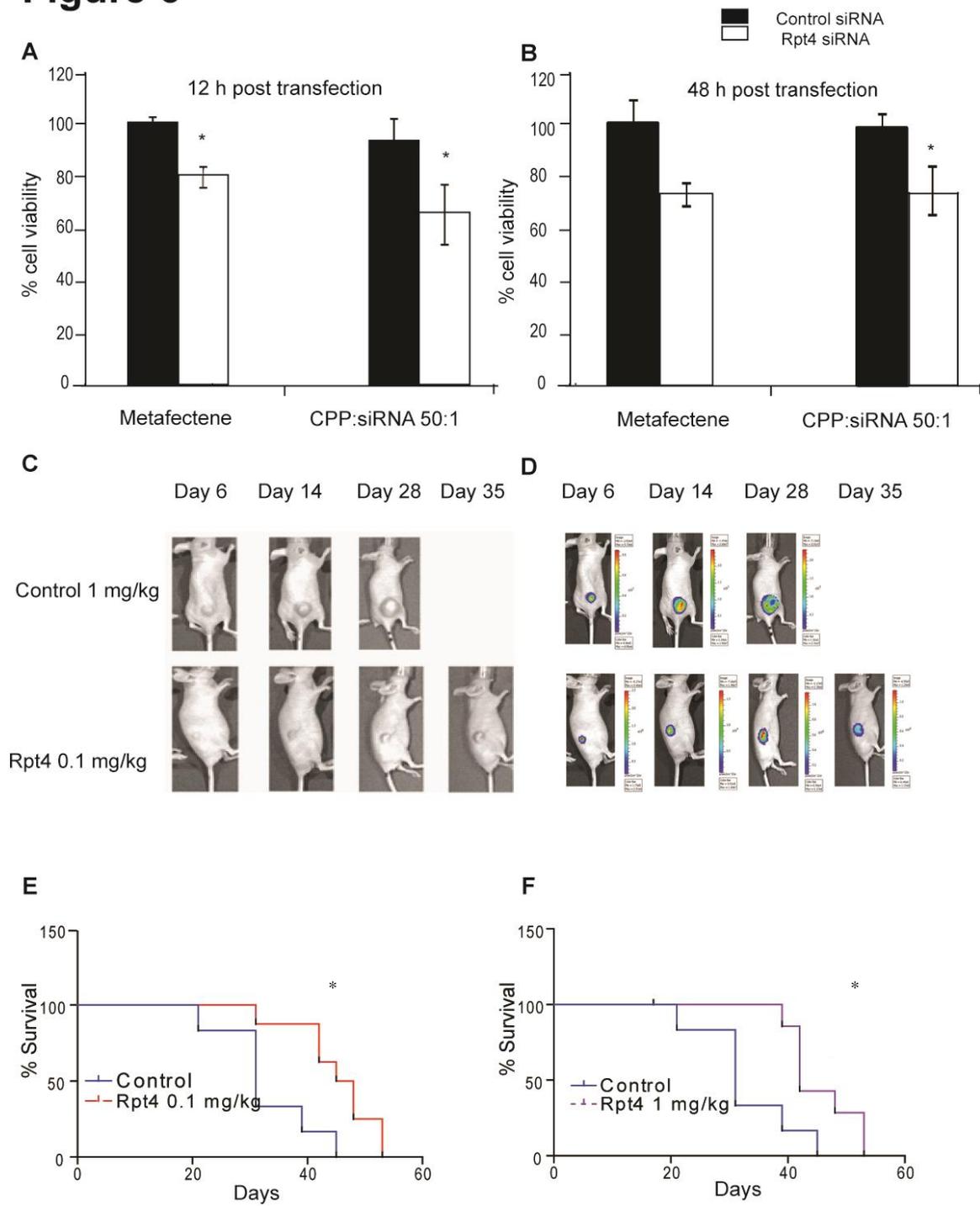


Table 1: Particle size and zeta potential of CPP:siRNA nanoparticles

R8:siRNA ratio	Size (nm)	PDI	Zeta potential (mV)
siRNA	478.63 ± 29.68	0.64 ± 0.20	-17.67 ± 4.53
9:1	461.33 ± 10.21	0.41 ± 0.04	7.40 ± 1.25
25:1	347.37 ± 19.74	0.37 ± 0.05	11.31 ± 2.71
50:1	237.87 ± 11.97	0.42 ± 0.04	12.63 ± 0.51

The average particle size, polydispersity index (PDI) and zeta potential (ZP) of the nanoparticles was measured using a Zetasizer (Nanoseries, Nano-ZS, Malvern Instruments,UK). Data is presented as the average of 5 measurements +/- standard deviation.