

E3 ligase cIAP2 mediates downregulation of MRE11 and radiosensitization in response to HDAC inhibition in bladder cancer

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Abstract

The MRE11/RAD50/NBS1 (MRN) complex mediates DNA repair pathways, including double-strand breaks induced by radiotherapy. Meiotic recombination 11 homolog (MRE11) is downregulated by histone deacetylase inhibition (HDACi), resulting in reduced levels of DNA repair in bladder cancer cells and radiosensitization. In this study, we show that the mechanism of this downregulation is post-translational and identify a C-terminally truncated MRE11, which is formed after HDAC inhibition as full-length MRE11 is downregulated. Truncated MRE11 was stabilized by proteasome inhibition, exhibited a decreased half-life after treatment with panobinostat, and therefore represents a newly identified intermediate induced and degraded in response to HDAC inhibition. The E3 ligase cellular inhibitor of apoptosis protein 2 (cIAP2) was upregulated in response to HDAC inhibition and was validated as a new MRE11 binding partner whose upregulation had similar effects to HDAC inhibition. cIAP2 overexpression resulted in downregulation and altered ubiquitination patterns of MRE11 and mediated radiosensitisation in response to HDAC inhibition. These results highlight cIAP2 as a player in the DNA damage response as a post-transcriptional regulator of MRE11 and identify cIAP2 a potential target for biomarker discovery or chemo-radiation strategies in bladder cancer.

Introduction

The Mre11/Rad50/Nbs1 (MRN) complex plays an integral role in the DNA damage response, by initiating signal transduction mechanisms to repair double strand breaks (DSBs) and by direct resection of damaged DNA in the homologous recombination (HR) and microhomology-mediated end-joining (MMEJ) repair pathways(1),(2). MRE11 in particular has a pivotal role as a functional exo- and endonuclease and is an emerging drug target in cancer(3),(4).

Although organ-confined muscle invasive bladder cancer is frequently treated by neoadjuvant chemotherapy followed by surgical removal of the bladder (cystectomy), radiotherapy with concurrent use of a radiosensitiser is a viable alternative to cystectomy. Concurrent chemoradiation is now a standard of care (www.nice.org.uk/guidance/ng2) with evidence of superior outcomes over radiotherapy alone(5), but available radiosensitising agents are generally too toxic for patients over 80 years. There is therefore an urgent need to find less systemically toxic agents which furthermore do not add to the toxicity of radiation in surrounding normal tissues for this increasingly elderly patient population. Histone deacetylase (HDAC) inhibitors exhibit low toxicity to normal cells(6), and the HDAC inhibitor panobinostat shows promise as a radiosensitiser *in vitro*(7). Panobinostat has not yet been used in bladder cancer but is FDA approved for use in multiple myeloma and currently in clinical trials for a range of solid tumours (www.clinicaltrials.gov). We hypothesised that panobinostat alone could be used as an effective radiosensitiser. Panobinostat downregulates MRE11(7) and other homologous recombination (HR) proteins, which results in decreased ability to repair DNA(6). The classic HDACi mechanism involves increased acetylation of histones, allowing transcription factors access to the promoters of genes which are up or downregulated, although increases in non-histone acetylation of DNA repair factors are also observed(8). To date HDAC inhibitors have shown efficacy, in some cases specific to individual classes of HDAC(9), in many diseases with a broad range of targets. This includes an increasing range of cancer subtypes which

have shown positive responses via various pathways, including altered gene transcription, chaperone regulation and cell death(10),(11),(12).

In addition to transcriptional downregulation of proteins in the DNA damage response, post-transcriptional mechanisms mediated by post-translational modification are also important in DNA repair pathways. Covalent linkages of ubiquitin, SUMO and NEDD are emerging as important modifications in DNA repair both via proteasomal degradation pathways and as signalling modifications(13). E2 and E3 ligases control these modifications, examples include RNF8 and RNF168 which can ubiquitinate various DDR proteins including NBS1(14),(15). A candidate identified in this study which may regulate the DDR via MRE11 downregulation is cellular inhibitor of apoptosis protein 2 (cIAP2). cIAP2 is a RING type E3 ligase in the IAP family and has previously been identified in a screen of E3 ligases affecting DNA repair(16),(17). IAP family members have been shown to have a pivotal role in intrinsic and extrinsic cell death pathways, and are key players in the regulation of NF- κ B signalling via the ripoptosome and the innate immune response(18). cIAP2 is a biomarker for acute lymphocytic leukaemia outcome (19), although the precise function and substrate specificity of cIAP2 is unclear due to redundancy with cIAP1 in cell death and NF- κ B regulation.

In this study, we demonstrate that treatment of bladder cancer cells with the HDAC inhibitor panobinostat results in post-transcriptional downregulation of MRE11 and formation of a previously uncharacterised truncated form of MRE11 which is degraded by the proteasome. cIAP2 is identified as an E3 ligase upregulated by HDAC inhibition, which downregulates MRE11 and therefore regulates the DNA damage response. This is an important new link between DNA repair pathways and the IAP proteins which points to a wider network of proteins involved in the DNA damage response and a potential for crosstalk between radiosensitisation and extrinsic cell death pathways.

Materials and Methods

Cell lines and drug treatments

RT112 (generous gift from Margaret Knowles, University of Leeds, obtained 2009) and T24 cells (DSMZ, 2011) were cultured in RPMI, 293T (ATCC, 2011) and CAL29 (DSMZ, 2011) cells were cultured in DMEM supplemented with 10% foetal bovine serum (FBS) and incubated in 10% CO₂ at 37°C. Cell lines were confirmed to be mycoplasma negative by PCR in October 2016 and used up to 10 passages from original stock. 293T cells were used for immunoprecipitations, all other experiments were carried out in bladder cancer cell lines. Cell lines were previously validated by short tandem repeat (STR) analysis (DNA Diagnostics Center, Fairfield, OH). 25 nM panobinostat (Selleck), 0 – 2.5 µM staurosporine (Sigma), 10 µM MG-132 (Merck) and 50 µM Z-VAD-FMK (Sigma) in DMSO were used and 0 – 8 Gy ionising radiation was administered with a GSR-D1 caesium-137 irradiator (Gamma Medical Services). Cycloheximide was added at 10 µg/ml after 24 Hr treatment with DMSO or PAN 25 nM. T24 cell lines overexpressing cIAP2, cIAP2 knockdown and MRE11 knockdown were generated as previously described(20) using 300 µg/mL G418 to select for cells overexpressing cIAP2 and puromycin (2 µg/ml) to select for cells expressing shcIAP2.

Plasmids and Transfections

T24 and RT112 cells were transiently transfected with pcDNA 3.3 containing N-terminal HA tagged FL-MRE11 or TR-MRE11 (aa 1–565) using Fugene (Promega) at a 3:1 Fugene:DNA ratio and cells harvested 24 – 48 Hr after transfections. cIAP2 constructs (amino acids FL: 1 – 604, B: 1 – 322, B and C: 1 – 529 C and R: 439 – 604) were cloned into pcDNA 4 using TOPO cloning (Life technologies) with C-terminal FLAG tags on CARD and RING domain constructs. shRNA to the 3' UTR of cIAP2 (Forward – 5'-GATCCGTTGAATAACTCTACAATGTTCAAGAGACATTGTAGAGTTATTCAACTTTTTTGAAA-3' Reverse – 5'-AGCTTTTCCAAAAAAGTTGAATAACTCTACAATGTTCAAGAGACATTGTAGAGTTATTCAACG-3') was cloned into pSilencer U.5 Puro (Ambion) according to manufacturers' instructions and transfected

with Fugene. 293T cells were transfected using polyethylenimine pH 7.4 (Sigma) at 3:1 PEI:DNA ratio and harvested after 24 – 48 Hr.

3'RACE and qRT-PCR

RNA was extracted using an RNeasy kit (Qiagen) and SYBR green mastermix (Applied Biosystems) used to amplify 1 µl cDNA generated from 2 µg total RNA using Bioline Sensifast reverse transcriptase kit. PCR amplification of MRE11 3'-cDNA ends was conducted using a 3'RACE kit (Life Technologies), with an *MRE11* gene specific primer (GSP): 5'-TCCAGACATTTTAAACCCAGA-3', positive control RNA provided in the kit and negative control without cDNA. qRT-PCR assays were performed using the 7500 fast RT-PCR instrument, and data analysed using standard $\Delta\Delta CT$ method(21) with StepOne software (Thermo Scientific). Transcript levels were normalised to two endogenous control genes, *ATP5B* and *SHDA*, and fold-change calculated relative to control samples.

Mass spectrometry

RT112 cells were harvested and lysed before running on an SDS-PAGE gel. After Coomassie staining, bands corresponding to FL or TR-MRE11 were excised and in-gel digestion carried out with trypsin or elastase as described previously(22) . Samples were analysed on an Orbitrap Q Exactive mass spectrometer coupled to an Ultimate 3000 LC (Thermo Scientific). Peak lists were generated using MSConvert (Proteowizard V3.0.5211 and searched using the Target Discovery Institute / Computational Biology Research Group (CBRG) in-house MASCOT search engine against the SwissProt database (version 20131208) for human proteins (20,353 sequences). The following parameters were used: trypsin or elastase enzymatic cleavages allowing for up to two missed cleavages (trypsin) or none (elastase), peptide mass tolerance of 10 parts per million, fragment mass tolerance 0.06 Da, fixed modification as a result of carbamidomethylation of cysteine, and variable modifications of oxidation of methionine and deamidation of asparagine or glutamine. The MASCOT Mowse score cut-off for confident peptide identification applied was 20.

Cell death analysis

An Abcam caspase activity kit (ab112130) was used to stain cells. Cells were analysed using a FACSsort instrument (BD) and FlowJo software.

Western blots and co-immunoprecipitations

Cell lysates were prepared in 50 mM HEPES, 100 mM NaCl, 10 mM EDTA, 1% Triton X-100, 4 mM Na pyrophosphate, 2 mM Na orthovanadate, 10 mM NaF, 50 mM B-glycerophosphate and mini-complete protease inhibitors (Roche). Total protein concentration was determined by BCA assay (Thermo) and 10–50 µg of protein analysed by SDS-PAGE. For immunoprecipitations 300 µg lysate was pre-cleared with protein G dynabeads (Pierce) at 4°C for 30 minutes. Lysates were incubated overnight with antibody then added to protein G beads for 1 Hr at room temperature. Beads were washed six times in PBS-Tween 0.05% and binding proteins eluted by boiling in 15 µl Laemmli buffer before SDS-PAGE. 5% of the total lysate was retained as the load fraction. HA pulldowns were carried out using anti-HA (YPYDVPDYA) conjugated magnetic beads (Thermo) pre-clearing as above, then incubating overnight at 4°C with anti-HA beads and eluting as above. Antibodies used were MRE11 – ab214 and ab30725 (Abcam), Nbs1 – NB100-143 (Bio-technique), RAD50 ab89 (Abcam), B-Actin – ab6276 (Abcam), Histone H3 AcLys14 – 7627 (Cell Signalling), cIAP2 58C7 – 3130S (Cell Signalling), cIAP1/2 – MAB3400 (Bio-technique), Anti-FLAG 9A3 – 8146P (Cell Signalling), Anti-HA – ab18181 (Abcam) and FK2 – B2500-0100 (Viva Bioscience).

Immunohistochemistry

Cell pellets were generated from RT112 and T24 cancer cell lines and fixed in 10% formalin before embedding in paraffin using the HistoStar™ Embedding Workstation (Thermo Fisher Scientific, US). 4 µm sections were cut with a microtome, mounted onto slides and manually stained for ab30725 using an Avidin/Biotin Blocking Kit (Vector Laboratories) and REAL Detection Systems (LSAB+) kit (Dako). Sections were stained for ab214 using the Bond Polymer Refine Detection kit (Leica

Microsystems) and the Leica Bondmax Autostainer. Slides were visualised using 400x magnification on the slide scanner Aperio CS2 with Aperio ImageScope software.

Ubiquitination assay

Ubiquitination assays were carried out as previously described(23). Lysates were incubated for 4 hr at room temperature with Nickel-NTA agarose (Qiagen), then beads were washed sequentially with ubiquitination assay buffers and protein eluted for 30 minutes at room temperature with imidazole buffer before SDS-PAGE and western blot analysis.

Colony formation assay

T24 cells and stable cell lines were plated in appropriate numbers in triplicate (600 – 2000) and irradiated at 0 – 6 Gy as previously described(20). After incubation for 7 – 10 days cells were fixed with 93% methanol 7 % acetic acid, then stained with Coomassie Brilliant Blue. Colonies were counted using the ColCount instrument (Oxford Optronix).

Comet assay

Cells were treated with 20 Gy ionising radiation and immediately trypsinised and kept on ice, or left for 4 Hr at 37°C then trypsinised. Cell suspensions were added to 1% low melting point agarose (Biorad) and layered on to slides pre-coated with 1% normal melting point agarose then set on ice. Slides were incubated in lysis buffer (2.5M NaCl, 100 mM EDTA, 10 mM Tris Base, 1% sodium lauryl sarcosinate, 1% triton X-100, 10% DMSO), pH 9.5 for 2 Hr, then soaked in TBE for 1 Hr prior to electrophoresis at 1.5V/cm for 30 minutes. After air-drying overnight slides were rehydrated with 1:10000 SYBR gold and air-dried again. Fifty cells per slide were analysed on a Nikon Eclipse 90i microscope using Komet software (Oxford Instruments).

Data information

Western blots are representative of three or more independent experiments. qRT-PCR was carried out in three technical replicates each for three biological replicates. P-values were calculated using unpaired T-test in GraphPad Prism, * indicates $p\text{-val} < 0.05$, mean and SEM is indicated by error bars. Densitometry of western blots was calculated using Image J software. Half-life was determined by fitting data to an exponential decay model in GraphPad prism. For clonogenic assays surviving fraction was calculated based on the number of colonies on non-irradiated plates. Data is presented as the log of the surviving fraction with error bars showing the SEM. For comet assays graphs represent 50 individual measurements per sample and SEM is indicated by error bars.

All data and materials are available from authors upon request.

Results

A C-terminally truncated form of MRE11 is induced by HDAC inhibition

A decrease in MRE11 levels after panobinostat (PAN) treatment is associated with radiosensitisation of bladder cancer cell lines, as lower levels of the MRN complex lead to decreased ability to repair DNA double strand breaks caused by ionising radiation(7). In the bladder cancer cell lines T24 and RT112 this decrease in MRE11 is observed 24 hours after treatment, along with an increase in the formation of an approximately 67 kDa truncated MRE11 (TR-MRE11) detected by western blot (Fig 1a, S1d,e). This truncated form of MRE11 was mapped by mass spectrometry and lacks the C-terminal region of the protein, which includes a DNA binding domain (Fig. S1a,b). In T24 cells TR-MRE11 is only detected 24 Hr after PAN treatment whereas RT112 cells already have high endogenous levels of TR-MRE11. An initial large increase in acetylated histone H3 (AcK10) is induced by HDAC inhibition, decreasing 8 – 24 Hrs after treatment at the time point when reduced MRE11 levels are observed. The transcript level of *MRE11* was not significantly decreased by either ionising radiation (IR), panobinostat treatment or a combination of the two in RT112 cells although expression was decreased in T24 cells with the combination of treatments (Fig 1b). This indicates that downregulation of MRE11 after PAN treatment occurs predominantly on a post-transcriptional level. In the RT112 cell line no alternative *MRE11* transcripts were detected which would result in a protein of 67 kDa, indicating that the truncation is formed post-transcriptionally rather than being a splice variant (Fig 1c). The later time point of the MRE11 decrease in comparison to the increase in acetylated histone H3 could therefore indicate the involvement of an intermediate which is transcriptionally regulated by HDAC inhibition. The moderate decrease of FL-MRE11 after PAN treatment accompanied by an increase in TR-MRE11 is also observed in the gemcitabine resistant isotype of the CAL29 cell line(24) although not detected in the parental cell line (Fig. 1d), and TR-MRE11 is formed upon overexpression of FL-MRE11 in 293T cells, showing that it is possibly induced in order to regulate overall levels of MRE11 protein (Fig S1c). TR-MRE11 is not detected with

antibody ab30725 targeting an epitope in the C-terminal domain 600–708 (MRE11 FL), however both forms are detected by antibody ab214 targeting to amino acids 182–582 (MRE11 FL + TR) of MRE11 (Fig 1e,f), and immunohistochemistry staining of cell pellets detected an increased amount of staining for both FL and TR MRE11 forms in RT112 compared to T24, whereas using an antibody that cannot detect TR-MRE11 the cell lines have a similar level of FL-MRE11. This corresponds to the protein levels seen in western blots (Fig 1a,g). Although a slight reduction in NBS1 binding is seen in T24 cells, the core MRN complex is not destabilised by treatment with PAN (Fig. 1h). This suggests complex dissociation is not a major driver for MRE11 downregulation as a result of the decrease in NBS1 levels. As TR-MRE11 is induced in concert with the downregulation of FL-MRE11 we hypothesised that it could represent a degradation intermediate or less stable form of FL-MRE11.

TR-MRE11 is degraded by the proteasome in response to panobinostat treatment

To investigate whether post-transcriptional downregulation of MRE11 is via the ubiquitin-proteasome pathway, RT112 and T24 cells were treated with the proteasome inhibitor MG-132. This resulted in a decrease in full-length MRE11 similar to the decrease seen after PAN treatment, but a stabilisation of TR-MRE11 was observed after treatment with PAN in both RT112 and T24 cells, with an increased truncation to full-length ratio in both cell lines (Fig 2a,b). This demonstrated that TR-MRE11 can be degraded by the proteasome and PAN potentiates both the formation of TR-MRE11 and degradation of the shorter protein. Chloroquine treatment had no effect on levels of MRE11, suggesting this is not via the autophagy/lysosome pathway (Fig 2, Fig. S2a). RT112 cells were treated with cycloheximide to measure the half-life of TR-MRE11 in RT112 cells. The calculated half-life of TR-MRE11 was reduced from 18 to 8 hours in PAN treated cells (Fig. 2c,d, Fig S2b), indicating decreased stability of the truncated product after PAN treatment. It is notable that FL-MRE11 is only modestly affected by cycloheximide until later time points as previously shown(25), suggesting it is

relatively stable once translated. TR-MRE11 could therefore represent a less stable intermediate which is more accessible to ubiquitination or the proteasome.

Identification of cellular inhibitor of apoptosis protein 2 (cIAP2) as an E3 ligase which binds MRE11

To investigate potential degradation mechanisms for MRE11 we screened a panel of E2 and E3 ligases previously implicated in the DNA damage response(16). This revealed a number of genes up- or downregulated by PAN treatment. The E3 ligase cIAP2 (*BIRC3*) was the most highly upregulated gene in RT112 and T24 cell lines (Fig. 3a). Other upregulated genes included *RNF168*, *Ube2h* and *TTC3*. Relative expression of *BIRC3* increased with a titration of PAN, although transcript levels dropped at a higher concentration in T24 cells, suggesting a feedback loop mechanism or adverse effects of a high concentration of drug, which was twice the working concentration (Fig 3b). This was recapitulated at the protein level with an immediate increase in cIAP2 levels sustained in both cell lines up to 24 Hr when downregulation of FL-MRE11 and appearance of TR-MRE11 is observed (Fig 3c, 1a, S3a,f). Transcript levels of two other members of the IAP family, *XIAP* and cIAP1 (*BIRC2*) which regulate cell death pathways showed *BIRC2* is upregulated after PAN treatment, but the dominant caspase regulator *XIAP* is unchanged with HDAC inhibition or IR. *BIRC2* was upregulated with IR alone, which is not seen with cIAP2 suggesting cIAP2 upregulation is primarily due to HDAC inhibition and not solely downstream of the DNA damage response (Fig 3d). cIAP2 is known to be promoted by ER/NF- κ B in a manner dependent on the histone acetyl transferase CBP(26), therefore this may be the mechanism of upregulation. We carried out FACS analysis to investigate apoptotic effects of HDAC inhibition by measuring caspase activity and western blots to visualise levels of cleaved PARP and saw no increase in either with PAN treatment (Fig S3b,c). Cells treated with PAN exhibited higher caspase activity and levels of cleaved PARP only in combination with staurosporine compared to untreated cells, suggesting apoptotic mechanisms can be initiated by other pathways after PAN treatment but not as a direct result of HDAC inhibition (Fig. S3a,b). Staurosporine

treatment results in an increase in cleaved PARP but not TR-MRE11, and as panobinostat has the opposite effect inducing TR-MRE11 but not cleaved PARP we conclude that TR-MRE11 is not primarily formed by apoptotic cleavage (Fig S3d,e). After treatment with PAN in combination with the caspase inhibitor Z-VAD-FMK a decrease in TR-MRE11 and stabilisation of FL-MRE11 was observed, suggesting caspases catalyse MRE11 cleavage (Fig 3e). These data taken with studies showing that cIAP1 and cIAP2 are poor caspase inhibitors compared with XIAP(27) suggests apoptotic mechanisms are not the main cause or function of upregulated cIAP2 after PAN treatment, but IAP and caspase related pathways may be involved in MRE11 regulation.

In 293T cells co-transfected with HA tagged FL-MRE11 (HA-FL-MRE11), or an HA TR-MRE11 construct lacking the MRE11 C-terminus (aa 1-565, HA-TR-MRE11) and full-length cIAP2, cIAP2 co-immunoprecipitates with both FL-MRE11 and TR-MRE11, showing it can directly bind or is part of a larger complex with MRE11 (Fig. 4a). When cells were treated with MG-132 a large amount of ubiquitinated cIAP2 was detected binding to both FL and TR-MRE11 after PAN treatment which may be due to increased levels of cIAP2 which can auto-ubiquitinate (Fig. 4b,c). Endogenous cIAP2 immunoprecipitated endogenous MRE11 both before and after PAN or MG-132 treatment (Fig. 4d). Deletion constructs of cIAP2 and MRE11 were used to map this interaction to the BIR domain of cIAP2 which was previously reported to mediate protein-protein interactions for substrates of the IAP family(28), which is consistent with TR-MRE11 being a cIAP2 substrate (Fig. 4e,f). The double band visible for cIAP2 in Fig. 4f may represent a modified form of cIAP2, or the less intense band could be endogenous cIAP2. BIR and BIR-CARD domain cIAP2 constructs bind MRE11 less well than FL-cIAP2, which may be a function of the necessity of RING domain dimerisation to facilitate cIAP2 binding substrates or may indicate multiple binding sites.

cIAP2 downregulates MRE11 via proteasomal pathways and increases ubiquitination of MRE11

To investigate effects of cIAP2 on MRE11 we transfected cIAP2 into T24 cells in increasing quantities. This resulted in decreased MRE11 levels comparable to that seen with PAN treatment (Fig. 5a).

Notably, in T24 cells, using an antibody recognising both cIAP1 and cIAP2 a decrease in cIAP1 relative to increasing cIAP2 levels is observed, agreeing with previous studies of the relationship between the two proteins and further supporting a function distinct from cell death mechanisms(29),(30). PAN treatment enhanced downregulation of MRE11 by cIAP2 in T24 cells, and the cIAP2-mediated decrease was abrogated by MG-132 treatment confirming this downregulation is proteasome-dependent (Fig. 5b, S4a). This effect was also seen in cells treated with 5 Gy (Fig. 5b, S4b) In T24 cells treated with MG-132, truncated MRE11 is visible therefore TR-MRE11 may be formed and quickly degraded by the proteasome in the absence of MG-132. cIAP2 overexpression is enhanced by PAN treatment, to a greater level than overexpressing the protein alone. This could be due to increased stability of cIAP2 as a dimer, as PAN increases endogenous levels of cIAP2. HA-FL-MRE11 was co-immunoprecipitated from 293T cells and bands corresponding to approximately the molecular weight of FL-MRE11 and TR-MRE11 were detected with the FK2 antibody targeting ubiquitin conjugated to proteins, along with an overall increase in higher molecular weight ubiquitinated species immunoprecipitated after cIAP2 transfection in the presence of MG-132 (Fig. 5c). This assay indirectly detects ubiquitinated proteins bound to MRE11 as well as ubiquitinated forms of MRE11 therefore an *in vivo* assay using his-tagged ubiquitin to immunoprecipitate proteins covalently modified with ubiquitin(23) was used. FL-MRE11 which appears to be monoubiquitinated was detected, consistent with a previous high-throughput study.(31) TR-MRE11 was also immunoprecipitated with his-tagged ubiquitin, and higher molecular weight species indicating multiple ubiquitinations observed (Fig 5d,e). PAN treatment increases the amount of FL-MRE11 conjugated to ubiquitin which is immunoprecipitated, and ubiquitinated TR-MRE11 is detected although PAN treatment has little effect on levels of these modified forms. An increased amount of FL-MRE11 modified with ubiquitin is also detected after cIAP2 transfection similarly to PAN treated cells. The amount of ubiquitinated TR-MRE11 increases after cIAP2 transfection, indicating ubiquitination of this species is promoted by cIAP2 though unaffected by PAN alone. This suggests PAN can mediate MRE11 truncation via upregulation of cIAP2, but cIAP2 can bring about

ubiquitination of TR-MRE11 without PAN treatment. This supports a model where MRE11 is either a substrate for cIAP2, or cIAP2 can prime MRE11 for processing by other E3 ligases, for example as a scaffold, and PAN potentiates the initial mono-ubiquitination and truncation of MRE11.

cIAP2 levels affect the radiosensitivity response to panobinostat

If cIAP2 is critical for MRE11 regulation after PAN treatment it should have an effect on radiosensitivity. We therefore generated stably overexpressing or cIAP2 shRNA knockdown T24 cells and carried out clonogenic assays to assess radiosensitivity and cIAP2 effects on DNA repair (Fig S5a,b). T24 cells are more radiosensitive after PAN treatment as previously shown(7), and a delay in DSB repair was measured by neutral comet assay (Fig. 6a,d). shcIAP2 cells do not show increased radiosensitivity after HDAC inhibition, or a significant difference in tail moment 4 hours after damage with PAN treatment compared to untreated (Fig 6b,e). cIAP2 overexpressing cells show increased radiosensitivity in response to PAN treatment and a delay in DNA repair (Fig 6c,f). This delay is moderate, which corresponds to the small decrease in MRE11 induced by PAN treatment, unlike the large repair delay induced in MRE11 knockdown cells (Fig S5c). Notably, shcIAP2 cells appear to have a slightly higher radiosensitivity and decreased gamma H2AX response compared to the wild type cell line, while overexpressing cells do not (Fig S5d,f). This could be due to differences in ability to degrade TR-MRE11 which may not be functionally active, or relating to other cIAP2 functions in NF- κ B signalling. Phospho-H2AX levels are higher than wild type in MRE11 knockdown RT112 cells and also in wild type RT112 cells treated with PAN at 4 Hours post-IR as DNA repair delay occurs and phospho-H2AX accumulates. Transfecting cIAP2 has a similar effect in wild type cells with phospho-H2AX present after 4 Hr, but does not affect repair in MRE11 knockdown cells suggesting the DNA repair delay function of cIAP2 is primarily via MRE11 regulated pathways (Fig S5e). As TR-MRE11 is formed upon downregulation of FL-MRE11 by PAN treatment, binds cIAP2 and is ubiquitinated in response to an increase in cIAP2 levels, a model is proposed where HDAC inhibition induces formation of TR-MRE11 and transcriptionally upregulates cIAP2, which can potentiate MRE11

ubiquitination, thus increasing MRE11 turnover and resulting in decreased ability to repair damaged DNA (Fig. 7).

Discussion

MRE11 is a mediator of several DNA damage response pathways(32), and downregulation of MRE11 is a target for radiosensitisation via impairing cancer cells ability to repair DNA damage. Although previous *MRE11* variants have been described that impact on DNA repair (33),(34),(35),(36), little has yet been reported on MRE11 post-translational modification or downregulation; indeed it has been reported to have a long half-life(25). As a protein essential for replication and telomere maintenance(37),(38), tightly controlling levels of MRE11 is an important strategy for the cell to maintain genetic stability and surveillance, and excess MRE11 could be as deleterious as low levels of the essential protein. We have discovered a truncated form of MRE11 (TR-MRE11) is formed when downregulation of the full-length protein is observed, and TR-MRE11 can be degraded by the proteasome. It is possible that the full-length protein is not in the correct conformation to be recognised by the ubiquitin-proteasome machinery and TR-MRE11 is more amenable to degradation. Previous immunohistochemistry (IHC) results show a correlation between high MRE11 expression in patient samples and a favourable response to radiotherapy in bladder cancer(39),(40). This is counterintuitive, as cells with more MRE11 are expected to proficiently repair radiation induced double strand breaks. If TR-MRE11 is functionally compromised or a step in the degradation of FL-MRE11, and TR-MRE11 is the overexpressed form detected in IHC studies, this would explain the discrepancy. The data here indicate a strategy to exploit TR-MRE11 as a more effective predictive biomarker.

The E3 ligase cIAP2 has mainly been investigated in studies on apoptotic pathways regulated by the IAP family(41), and as both a positive and negative regulator of NF- κ B pathways(42) and the innate immune response(43). A common feature of these studies is that redundancy between cIAP1 and cIAP2 is apparent, and cIAP2 plays a minor role compared to cIAP1 and XIAP in apoptosis. cIAP2 has a larger nuclear fraction than cIAP1, and is detected in lower endogenous levels, hinting at unique roles which have not yet been discovered(44). Nuclear cIAP1 predicts poor prognosis in bladder

cancer(45), raising the possibility of overlapping functions with cIAP2 regarding MRE11, dependent on the localisation of the two proteins. There is also an important difference in IAP response to Smac mimetics(29), which target XIAP and re-activate apoptotic pathways with some success as an anti-cancer treatment. cIAP2 levels increase in response to degradation of cIAP1 induced by Smac mimetics(30), therefore it appears to be less sensitive to Smac mimetic-mediated degradation, and Smac mimetics can actually stabilise and activate the E3 activity of cIAP2. This raises the intriguing possibility of using Smac mimetics as radiosensitisers by activating cIAP2 towards MRE11, and indeed the Smac mimetic BV6 has been shown to act as a radiosensitiser(46). Although in this study we observe cIAP2 binding to MRE11, there is a possibility that this is via another binding partner in a larger complex and cIAP2 acts as a scaffold, which has been seen in studies of cIAP1 and XIAP activity(29). Further work could confirm MRE11 as a direct ubiquitination target of cIAP2, and what form this ubiquitination takes. As well as multiple ubiquitination we observe monoubiquitination of MRE11, which has been reported in several high throughput studies, and it would be interesting to see if this has signalling functions, such as the SUMOylation of Nbs1 described previously(47).

Although here we show TR-MRE11 is amenable to degradation by the proteasome, and identify cIAP2 as a potential E3 ligase which downregulates MRE11, we have not fully investigated the mechanism by which MRE11 is truncated in this study. A putative caspase cleavage site(48) is situated near the position where MRE11 is truncated, this and a reduction in TR-MRE11 after caspase inhibition could indicate that caspases regulate the truncation of FL-MRE11. As cIAP2 contains a caspase recruitment and activation domain (CARD) and is known to bind caspases in a non-inhibitory manner(27) there is the possibility of linkage between cIAP2 and the formation of the MRE11 truncation via caspase pathways, for example if DNA repair is not required when caspases are activated for apoptosis the truncation may accelerate MRE11 degradation. The cIAP2-MALT1 fusion protein has been shown to play a role in many cancers, via the paracaspase function of MALT1(49) and cIAP2 is often mutated in cancers where ATM is mutated, further suggesting a role in regulation of DNA damage and repair(19). Proteasomal inhibition does not prevent the formation of

the truncation, confirming it is generated by a protease distinct from the proteasome. The model proposed by us in this paper could be refined by further examining the effects of caspases in the formation of truncated MRE11. As TR-MRE11 is induced by HDAC inhibition but also has a shorter half-life, it appears that turnover of MRE11 is mediated by HDAC inhibition. There is an equilibrium between full-length, truncated, and degraded MRE11, which can be modulated by drugs such as PAN, and here we show that the initial truncation of MRE11 is dependent on PAN and subsequent degradation reliant on cIAP2.

In conclusion, we present a model of MRE11 downregulation via proteasomal degradation of a C-terminally truncated intermediate. The E3 ligase cIAP2 is upregulated by panobinostat, and binds both full-length and truncated MRE11. Cells with reduced cIAP2 levels no longer have an increased sensitivity to radiation after panobinostat treatment, suggesting this pathway plays a major role in the radiosensitisation response to HDAC inhibition. cIAP2 is also an interesting new player in the DNA damage response, and there is potential to further define the mechanisms by which it regulates radiosensitisation. Due to the availability of Smac mimetics which may activate cIAP2 activity, we suggest that these compounds may be well suited for the clinical development of radiosensitisers in bladder cancer, and MRE11 turnover could represent a promising new target for chemoradiation.

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Figure Legends

Figure 1 - A) Protein levels of core Mre11/Rad50/Nbs1 (MRN) complex members and acetylated histone H3 in RT112 and T24 cells after treatment with 25 nM panobinostat. TR-MRE11 is detected at approximately 67 kDa after 24 Hr treatment in T24 cells and is present in RT112 cells at all time points. B) Relative gene expression measured by transcript levels of MRE11 is not significantly changed after 25 nM panobinostat, 4 Hr after 5 Gy ionising radiation or a combination of the two in RT112 cells and is significantly changed in T24 cells treated with the combination of drug and IR. Relative quantitation (RQ) values were normalised to cells treated with DMSO. C) Amplification of MRE11 3' cDNA in RT112 and T24 cell lines using gene specific primers (GSP), +ve – positive control, -ve, negative control D) MRE11 levels in CAL29 and gemcitabine-resistant CAL29 cell lines treated with 25 nM panobinostat. E) Comparison of antibody detection of FL and TR-MRE11 by ab214 (12D7, aa 182 – 582) and FL-MRE11 only by ab30725 (aa 600 – C-terminus). F) Schematic of MRE11 domains – red line denotes site of truncation. G) Immunohistochemistry staining of either FL (ab30725) or FL + TR MRE11 (ab214) in RT112, T24 and stable MRE11 knockdown T24 cells. H) Immunoprecipitation of MRN complex with ab214 (IP: FL and TR-MRE11 ab214), control lane - non-specific mouse IgG used for IP.

Figure 2 - A) Protein levels of FL and TR-MRE11 after 25 nM panobinostat treatment and 10 μ M MG-132 treatment for 4 Hr in RT112 and T24 cells. B) Quantitation of intensity of bands in A, normalised relative to DMSO control treated cells and TR-MRE11:FL-MRE11 ratio at each time point. C) FL and TR-MRE11 levels after 10 μ g/ml cycloheximide treatment over a time course up to 12 hours in RT112 cells. D) Half-life determination of TR-MRE11 in (C) after cycloheximide treatment plus or minus 25 nM panobinostat treatment 24 Hr prior to CHX, all time points were normalised to cells untreated with cycloheximide.

Figure 3 – A) Fold change of E2 and E3 ligase transcript levels after treatment with 25 nM panobinostat in RT112 and T24 cells, RQ values are relative to cells treated with DMSO only. Dotted

line indicates genes upregulated by more than twofold. B) cIAP2 transcript levels increasing with a titration of panobinostat and, C) cIAP2 protein levels. D) Transcript levels of XIAP, cIAP1 and cIAP2 following treatment with 25 nM panobinostat, 4 Hr after 5 Gy ionising radiation or both treatments. E) Western blot of protein levels after 24 and 48 Hr treatment with 50 nM panobinostat and 50 nM Z-VAD-FMK alone and as a combination.

Figure 4 - A) Co-immunoprecipitation of transfected full-length cIAP2 by HA tagged FL-MRE11 or TR-MRE11. WCE – Whole cell extract. HA immunoprecipitation of cIAP2 after PAN and MG-132 treatment with B) HA-FL-MRE11, C) HA-TR-MRE11. D) Endogenous immunoprecipitation of cIAP2 and MRE11. Line indicates cIAP2, lower band is cIAP1 E) Domain structure of cIAP2 and deletion constructs, FL – full length, B – baculoviral induced repeat (BIR) domain, BC – BIR and caspase activation and recognition (CARD) domain, CR – CARD and RING finger domain. F) Immunoprecipitation of cIAP2 by HA tagged FL-MRE11 and BIR domain constructs. * indicates IgG bands.

Figure 5 - A) cIAP2 overexpression (0, 2.5, 5 and 10 μ g transfected) in T24 cells and MRE11 protein levels. MRE11 ab30725 - FL-MRE11, MRE11 ab214 - both FL and TR-MRE11. B) cIAP2 overexpression in T24 cells treated with DMSO control or 25 nM panobinostat and 10 μ M MG-132 with or without 5 Gy ionising radiation treatment. C) Immunoprecipitation of HA tagged FL-MRE11 transfected into 293T cells blotted for mono or poly-ubiquitinated species with FK2 antibody. D) His-tagged ubiquitination assay for HA-FL-MRE11 or HA-TR-MRE11 in 293T cells, cells were transfected with a combination of his-ubiquitin and HA-MRE11 and treated with DMSO or PAN 24 Hr prior to assay E) Cells transfected with HA-MRE11 as in D with or without co-transfection of cIAP2. All cells were treated with 10 μ M MG-132 prior to assay * indicates IgG bands.

Figure 6 – Clonogenic survival and neutral comet assays for A) T24, B) KD cIAP2 T24 (shcIAP2) or C) overexpressing cIAP2 (cIAP2 Over) T24 cell lines plus or minus 25 nM panobinostat treatment 24 Hr prior to irradiation. Values are the result of three independent experiments and survival curves were

fitted using the linear quadratic (LQ) model. D-F) Neutral comet assays for T24-based cell lines in A-C. Neutral comet assays prepared immediately after 20 Gy damage or with 4 Hr repair time before assay with and without 25 nM panobinostat treatment.

Figure 7 - *Model for regulation of a truncated form of MRE11 by cIAP2 ubiquitination after panobinostat treatment. HDAC inhibition results in an increase in truncated MRE11 and an increase in cIAP2 transcription. High levels of cIAP2 transcription contribute to the downregulation of MRE11 and result in an increased ubiquitination of MRE11, which results in a radiosensitisation due to decreased ability to repair DNA.*