Parallel High Throughput RNA interference Screens Identify PINK1 as a Potential Therapeutic Target for the Treatment of DNA Mismatch Repair Deficient Cancers

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Abstract

Synthetic lethal approaches to cancer treatment have the potential to deliver relatively large therapeutic windows and therefore significant patient benefit. To identify potential therapeutic approaches for cancers deficient in DNA mismatch repair (MMR), we have carried out parallel high throughput RNA interference screens using tumour cell models of MSH2 and MLH1-related MMR deficiency. We demonstrate that silencing of the PTEN-induced putative kinase 1 (PINK1), is synthetically lethal with MMR deficiency in cells with either *MSH2*, *MLH1* or *MSH6* dysfunction. Inhibition of PINK1 in an MMR deficient background results in an elevation of reactive oxygen species and the accumulation of both nuclear and mitochondrial oxidative DNA lesions, which likely limit cell viability. Therefore, PINK1 represents a potential therapeutic target for the treatment of cancers characterised by MMR deficiency caused by a range of different gene deficiencies.

Introduction

The DNA mismatch repair (MMR) pathway plays a key role in the maintenance of genomic stability. One of the best characterised functions of MMR is the postreplicative repair of DNA polymerase errors such as base-base mismatches or insertion/deletion mismatches. MMR also has a role in the repair of DNA damage, such as oxidised bases including 8-oxo-guanine. Given this role, it is perhaps unsurprising that defects in the genes that mediate MMR cause a 'mutator' phenotype and predisposition to a range of tumour types, including colorectal, gastric, endometrial and bladder cancer (1). For example, the hereditable disorder, Lynch Syndrome (LS), (Hereditary Non Polyposis Colon Cancer (HNPCC)), is associated with germline loss of function mutations in the MMR tumour suppressor genes *MLH1, MSH2, PMS2* and *MSH6* (2). In addition to this familial syndrome, MMR deficiency is present in 15-17% of all colorectal cancer cases and a common cause of non-familial MMR deficiency is biallelic hypermethylation of the *MLH1* promoter that results in a reduction of MLH1 expression (3).

The concept of exploiting synthetic lethal/sickness (SSL) relationships has been proposed as a potential route to identifying novel therapeutic approaches to cancer (4). A synthetic lethal relationship between two genes or proteins exists when loss of function of either protein alone is compatible with cell viability but simultaneous loss of both proteins causes cell death or inhibition (4, 5). This concept can be used to design therapeutic approaches that target the cancer cell-specific loss of tumour suppressor proteins. For example, if a tumour suppressor gene and a second gene are synthetically lethal or sick, inhibition of the second gene would selectively kill or inhibit cancer cells having loss of the tumour suppressor (4, 6). The utility of this approach has been vindicated by the demonstration of the clinical applicability of synthetic lethal targeting of BRCA1 or BRCA2 deficient tumours with PARP inhibitors (7-9). Similarly, synthetic lethal relationships have been identified that involve specific MMR genes (10) suggesting that the synthetic lethal paradigm could be applied to MMR deficiency. For example, loss of MSH2 function is synthetically lethal with inhibition of the proofreading DNA polymerase, POLB, whilst MLH1 deficiency is synthetically lethal with inhibition of a different DNA polymerase, POLG (10).

Intriguingly, whereas POLB inhibition is selective for MSH2 deficiency, it does not cause cell death in MLH1 deficient cells and the effect of POLG inhibition is similarly MLH1 specific (10). So far, candidate targets have not been identified that are synthetically lethal with a range of MMR gene deficiencies. Such targets would have a broader utility than synthetic lethalities that are specific for particular MMR genes.

With the aim of identifying novel candidate therapeutic targets for cancers deficient in the MMR pathway, we carried out parallel siRNA screens in MSH2 or MLH1 deficient and proficient tumour cell models. This analysis and subsequent validation work identified PINK1, a mitochondrial protein kinase as a potential therapeutic target for tumours with either MSH2, MLH1 or MSH6 deficiency.

Materials and Methods

Cell lines and reagents

The human endometrial cell lines HEC59 and HEC59+Chr2 were the kind gift of Dr. T. Kunkel (NIEHS, NC, USA). HEC59+Chr2 and HEC59 cells were grown in DMEM F12 supplemented with FCS (10% v/v), glutamine and antibiotics. HEC59+Chr2 cells were maintained under selective pressure of 400 μg/mL geneticin (G418 sulfate). The human colon cancer cell line HCT116 and HCT116+Chr3 were the kind gift of Dr. A. Clark (NIEHS, NC, USA) and were grown in McCoys 5A supplemented with FCS (10% v/v), glutamine, and antibiotics. HCT116+Chr3 cells were maintained under selective pressure of 400 μg/mL geneticin (G418 sulfate). The human colon cancer cell line DLD1 and DLD1+Chr2 were the kind gift of Dr. T. Kunkel (NIEHS, NC, USA) and were grown in DMEM supplemented with FCS (10% v/v), glutamine and antibiotics. DLD1+Chr2 cells were maintained in 400 μg/mL geneticin (G418 sulfate). All cells were expanded for two passages, and cryopreserved. All experiments were performed with cells of passage of <14. These cell lines were authenticated based on viability, morphologic inspection and Mycoplasma tested. Selenomethionine was purchased from Sigma (UK).

Measurement of 8-OHdG

Mitochondrial and nuclear DNA were extracted using the mitochondrial DNA isolation kit (ab65321, Abcam) and digested with nuclease P1. A commercially available ELISA kit from Cayman Chemicals (MI, USA) was used to determine levels of 8-OHdG in isolated DNA. The assays were performed according to the manufacturers instructions. The 8-OHdG standard (0.0103–30 ng/ml) or 5 ⌈g DNA from cells was incubated with an anti-mouse IgG-coated plate with a tracer consisting of an 8-OHdG-enzyme conjugate. The assay was normalized by an equal amount of DNA used for each sample. Addition of a substrate to replicate samples was followed by measurement of absorbance at 412 nm. Standard curves were calculated for all reactions with serial dilutions of 8-OHdG standard to calculate reaction efficiency. Samples were assayed in triplicate.

Measurement of mitochondrial membrane potential

We used the MitoProbe $DiIC_1(5)$ assay kit (Invitrogen) according to the manufacturers instructions. This assay employs the cell-permeable cationic cyanine dye, $DiIC_1(5)$. The fluorescence intensity is proportional to the accumulation of the dye in mitochondria with active membrane potentials. Carbonyl cyanide m- chlorophenyl hydrazone (CCCP) was used as a positive control for disruption of the mitochondrial membrane potential.

Measurement of reactive oxygen species (ROS)

The OxiSelect ROS assay kit (Cell Biolabs) was used according to the manufacturers instructions. This assay employs the cell-permeable fluorogenic probe, 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA). The fluorescence intensity is proportional to the ROS levels within the cell cytosol.

siRNA transfections

Cells were transfected with short interfering RNA (siRNA) (Qiagen, UK or Dharmacon, UK). For 96-well plate-based cell viability assays, cells were transfected with individual siRNA using

Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. As a control for each experiment, cells were left un-transfected or transfected with a non-targeting Control siRNA (5'-CATGCCTGATCCGCTAGTC-3', Qiagen, UK) and concurrently analysed. Cell viability was measured five days after transfection using the 96-well plate CellTiter-Glo Luminescent Cell Viability Assay kit (Promega) according to the manufacturer's instructions. For clonogenic assays, exponentially growing cells were seeded at various densities in six-well plates. Cells were transfected with siRNA as before. Cell medium was replaced every four days. After ten to fourteen days, colonies were fixed in 10% trichloroacetic acid and stained with sulforhodamine-B. Surviving Fractions were calculated as the ratio of the plating efficiency (PE) of gene-specific siRNA transfected cells, divided by the PE of control siRNA transfected cells, where PE = colonies counted/cells plated. All transfections were carried out in triplicate.

siRNA screening

We used the siKINOME SMARTPool siRNA library (ThermoFisher), encompassing 779 siRNAs targeting protein kinases and kinase-related genes. In brief, cells were transfected with library siRNAs in a 96 well plate format. After seven days continuous culture, cell viability was estimated by use of the Cell TitreGlo reagent (Promega). Luminescence readings from each well were log transformed and normalized according to the median signal on each plate and then standardized by use of a Z score statistic, using the Median Absolute Deviation (MAD) to estimate the variation in each screen. Screen data was processed in this way using the CELLHTS2 package (11).

Protein analysis

Cell pellets were lysed in 20 mmol/L Tris (pH 8), 200 mmol/L NaCl, 1 mmol/L EDTA, 0.5% (v/v) NP40, 10% (v/v) glycerol, and protease inhibitors. Lysates were electrophoresed on Novex precast gels (Invitrogen, UK) and immunoblotted using the following antibodies: anti-MSH2 (Ab-1, Calbiochem, UK), anti-MLH1 (ab9144, AbCam), anti-CKMT2 (ab55963, AbCam), anti-PCK2 (ab77047, AbCam), anti-PCNA (SC7907, Santa-Cruz), anti-Cytochrome C (ab53056, AbCam), anti-PARP1 (Cell Signaling), anti-γH2AX (Milipore) and anti-β-tubulin, (T4026, Sigma, UK). This

was followed by incubation with anti-IgG-horseradish peroxidase and chemiluminescent detection (SuperSignal WestPico Chemiluminescent Substrate, Pierce, UK). Immunoblotting for β-tubulin was used as a loading control.

Quantitative RT-PCR (qRT-PCR)

Total RNA from cell lines was extracted from cells using Trizol (Invitrogen) according to manufacturers instructions. cDNA was synthesized using Omniscript Reverse Transcriptase System for RT-PCR (Qiagen) with oligo dT as per manufacturer's instructions. Assay-on-Demand primer/probe sets were purchased from Applied Biosystems. Real-Time qPCR was performed on the 790DHT Fast Real-Time PCR System (Applied Biosystems), using *GAPDH* as an endogenous control. Standard curves were calculated for all reactions with serial dilutions of control cDNA to calculate reaction efficiency. Gene expression was calculated relative to expression of *GAPDH* endogenous control, and adjusted relative to expression in control cDNA. Samples were quantified in triplicate

Results

MMR siKinome screens

To identify synthetic lethal interactions with mismatch repair deficiency, we performed parallel high-throughput viability screens using a library of short interfering RNAs (siRNAs) targeting 779 kinases and kinase-related proteins. We selected this subset to screen because of the key roles kinases play in a number of biological processes and also due to the inherent pharmacological tractability of this protein superfamily. To assess synthetic lethal interactions, we used isogenic models of MLH1 or MSH2 deficiency. To model MLH1 deficiency, we used the previously characterised human colon adenocarcinoma cancer cell line HCT116, which carries a homozygous mutation of the *MLH1* gene (12) and compared this to the MLH1 proficient HCT116+Chr3 cell line; transfer of human chromosome 3 into HCT116 cells results in the expression of functional MLH1 (13). To model MSH2 deficiency, we used the previously characterised human endometrial cell line HEC59, which harbours two different loss-of-function

MSH2 nonsense mutations (14). As a comparator for HEC59 cells, we used the MSH2 proficient HEC59+Chr2 cell line; transfer of human chromosome 2 into this cell line results in the expression of functional MSH2 (12). We screened each of these four cell lines with SMARTpool siRNAs that systematically target each of the 779 known and putative kinases. Each SMARTpool was composed of four distinct siRNA species targeting different sequences of the target transcript. Cells were transfected with siRNA and after seven days continuous culture, cell viability in each well was estimated by use of a luminescent assay measuring cellular ATP levels. These screens were repeated in triplicate and comparison of data from each screen revealed this approach to be highly reproducible, with r_2 correlation coefficients between screen replicas of >0.75. To identify loss of viability effects in each cell line, normalised luminescence readings from each well were log transformed and then corrected data according to median plate readings, to account for plate-to-plate variation. To allow data to be compared between the different matched paired-cell lines, plate-centered data from each cell line was standardised by the use of a Z score statistic, where Z=0 represents no effect on viability and negative Z scores represent loss of viability (Figure 1B & C). We compared the Z scores for each isogenically matched pair of cell lines to identify MMR selective effects, referred to as ΔZ, where ΔZ for each gene was equivalent to Z (MMR deficient line) subtracted from Z (MMR proficient line) (Figure 1B & C).

PINK1 silencing is synthetically lethal with MMR deficiency in a range of cellular models

To further validate the results observed in the primary screens, we performed a secondary screen with the top hits from both screens, using the same siRNA SMARTpools used in the original screen. Figure 2A &B illustrate the 20 most significant hits from each of the validation screens. Whilst a number of MLH1 or MSH2-specific effects were identified, we did note that silencing of the *PTEN induced putative kinase 1* gene (*PINK1*) was associated with significant selective lethality in both the MSH2 and MLH1 deficient cell lines. To establish the generality of these observations, we tested the PINK1 synthetic lethal interaction in the two matched paired cell lines used in the screens as well as an isogenic model of MSH6 deficiency. Here we used DLD1 colon carcinoma cells, which carry frameshift mutations in both alleles of *MSH6* and their MSH6

proficient comparator DLD1+Chr2 (Figure 3A). *PINK1* silencing by siRNA was selectively lethal with MMR deficiency in all three models (HEC59, HCT116 and DLD1). The two siRNAs targeting *PINK1* that generated the most significant synthetic lethal effects were also shown to cause synthetic lethality in all the MMR deficient cell models when used in clonogenic assays, therefore minimising the likelihood of an off target effect being responsible and also the possibility that the effects observed were particular to the Cell Titer Glo assay used thus far (Figure 3B, C & D). Given that MMR deficiency causes a mutator phenotype, it was possible that genetic drift between HEC59 and HEC59+Chr2 cells and HCT116 and HCT116+Chr3 cells could have explained the PINK1 synthetic lethalities we observed. To address this issue, we transfected HEC59+Chr2 cells with a combination of MSH2 and PINK1 siRNA. This combination elicited synthetic lethality, as did the combination of MLH1 and PINK1 siRNA in HCT116+Chr3 cells (Figure 3E), suggesting that mutations secondary to MMR deficiency were unlikely to explain the effects we observed. Confirmation of *PINK1* gene silencing by siRNA was performed by quantitative real time PCR (Supplementary Figure 1A).

PINK1 silencing leads to an accumulation of 8-oxoG in both mitochondrial and nuclear DNA in MMR deficient cells

PINK1 limits oxidative stress-induced apoptosis by suppressing cytochrome c release from mitochondria (15). We have previously demonstrated that synthetic lethality with MMR genes can be achieved by causing oxidative DNA damage in a MMR deficient background (10, 16). More specifically, MSH2 deficiency is synthetically lethal with genetic changes that result in an accumulation of 8-oxoG lesions in nuclear DNA (one of the more common oxidized DNA bases that are caused by oxidative damage), whilst MLH1 deficiency is synthetically lethal with genetic changes that cause mitochondrial DNA 8-oxoG accumulation (10). These effects are perhaps best explained by the role MMR proteins have in repairing oxidised DNA lesions such that lethality ensues in MMR deficient cells when these lesions are not repaired ((10) and references therein). Given the known function of PINK1, we hypothesised that the synthetic lethal interaction observed between PINK1 silencing and MMR deficiency, could be due to an increase in the

persistence of oxidative DNA damage. To assess this we transfected MMR proficient, MSH2 deficient or MLH1 deficient cells with PINK1 siRNA and then measured the formation of nuclear and mitochondrial 8-oxoG levels 72 hours after siRNA transfection using an ELISA (Figure 4A & B, Supplementary Figure 2). Increased 8-oxoG levels were observed in both the mitochondrial and nuclear DNA fractions from MSH2 deficient cells (Figure 4A) and also MLH1 deficient cells (Figure 4B) transfected with *PINK1* siRNA, whereas no significant accumulation of this lesion was observed in similarly transfected MMR proficient cell lines. This suggested that an accumulation of oxidative DNA damage could explain the synthetic lethal interaction between MMR deficiency and PINK1 silencing. We further examined the formation of 8-oxoG levels over time. Initially after PINK1 siRNA transfection,(48 hrs) 8-oxoG levels were seen to be elevated in both MSH2 deficient and proficient cells (Supplementary Figure 3A). However, 72 hours after PINK1 siRNA transfection, 8-oxoG levels were decreased in the MSH2 proficient cells but continued to rise in MSH2 deficient cells (Supplementary Figure 3A). These observations suggested that in both MMR deficient and proficient cells, inhibition of PINK1 initially leads to the formation of 8-oxoG lesions but these are eventually removed in MMR proficient cells but not MMR deficient cells. It seems likely that the persistence of 8-oxoG lesions in MMR deficient cells eventually reach a threshold that is incompatible with cellular viability.

Previously, PINK1 has been reported to be localized to the mitochondria (17). Therefore it was unclear why the PINK1/MMR synthetic lethalities resulted in an accumulation of 8-oxoG in not only the mitochondrial DNA but also in the nucleus. We hypothesised that silencing of PINK1 might disrupt mitochondrial membrane potential and that this mitochondrial dysfunction could result in an increase in oxidative stress that ultimately affected both mitochondrial and nuclear DNA. To address this we stained cells with the fluorescent lipophilic cyanine dye $DiIC_1(5)$. $DiIC_1(5)$ penetrates the cytosol of eukaryotic cells and accumulates primarily in mitochondria. The extent of mitochondrial accumulation of $DiIC_1(5)$ is dependent upon mitochondrial membrane potential and can be monitored by measuring $DiIC_1(5)$, where a loss of membrane potential results in loss of fluorescent signal (18). As a positive control, cells were treated with either

DMSO or 50⌠M carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a drug which is known to disrupt mitochondrial membrane potential. As expected, decreased $DiIC_1(5)$ fluorescence was observed in CCCP treated cells (Figure 4C). Interestingly $DiIC_1(5)$ fluorescence was significantly decreased in PINK1 silenced cells (P□0.0297), in comparison to control siRNA transfected cells (Figure 4C), strongly suggesting that inhibition of PINK1 decreases mitochondrial membrane potential perhaps resulting in the accumulation of oxidative damage in both the nucleus and the mitochondria.

We next explored whether PINK1 silencing in combination with MMR deficiency results in loss of cellular viability by apoptosis. Cleavage of PARP1 is a recognised marker of apoptosis induction. PARP1 cleavage was not observed in the Control siRNA transfected cells (Figure 4D). Significantly, seventy-two hours post PINK1 siRNA, increased cleavage of PARP1 was observed in the MSH2 deficient HEC59 cells, in comparison to the MSH2 proficient HEC59+Chr2 cells (Figure 4D & E). This indicated that the loss of viability observed upon MSH2 deficiency combined with PINK1 depletion, is associated with the induction of apoptosis. While the persistence of 8-oxoG lesions represented a potential mechanism explaining the MMR/PINK1 synthetic lethality, it was still possible that the elevated levels of oxidative damage observed were coincidental. To address this issue, we attempted to abrogate the synthetic lethal effect by addition of the antioxidant, selenomethionine. Addition of selenomethionine to tissue culture media has previously been shown to significantly reduce the level of 8-oxoG lesions (16, 19). We treated the PINK1 siRNA transfected MSH2 deficient and proficient cells with vehicle (DMSO) or with 1 μM selenomethionine, 24hr post transfection, for 72 hrs and then estimated PARP1 cleavage, cell viability and 8-oxoG levels. Selenomethionine treatment rescued the induction of apoptosis (Figure 4E), substantially reduced 8-oxoG accumulation in MSH2 deficient cells transfected with PINK1 (Supplementary Figure 3B) and rescued the MSH2/PINK1 synthetic lethality (Supplementary Figure 3C). Taken together, this suggests that oxidative damage most likely underlies the MMR selectivity of PINK1 silencing.

It has previously been shown that lethality caused by an accumulation of 8-oxoG lesions is characterized by an increase in single strand break (SSB) formation, which can ultimately lead to the formation of potentially lethal double strand breaks (DSBs) (20). To address this, we examined formation of nuclear DSBs in PINK1 silenced MSH2 proficient and deficient cell lines. The formation of nuclear DSBs can be efficiently monitored by the detection of γ-H2AX expression. Silencing of PINK1 resulted in the expression of γ-H2AX, ninety-six hours after transfection in MSH2 deficient cells, suggesting an increase in DSB formation (Supplementary Figure 3D). Significantly, addition of selenomethionine caused a reduction in this expression of γ-H2AX (Supplementary Figure 3D).

MMR deficiency is synthetically lethal with silencing of a number of mitochondrial kinases

Having demonstrated that silencing of the mitochondrial kinase PINK1 leads to an accummulation of 8-oxoG in MMR deficient cells (Figure 4A & B), we addressed the possibility that MMR synthetic lethality could be elicited by targeting other mitochondrial kinases. Analysis of the siRNA screen data, combined with gene annotation indicated that among the top scoring synthetic lethal hits in the MMR siKinome screens were a number of mitochondrially located proteins (21) (Table 1), including CKMT2 and PCK2. CKMT2, the mitochondrial creatine kinase, is responsible for the transfer of high-energy phosphate from mitochondria to the cytosolic carrier, creatine (22). PCK2 (phosphoenolpyruvate carboxykinase) catalyzes the conversion of oxaloacetate to phosphoenolpyruvate in the presence of GTP (23). Further validation experiments indicated that silencing *CKMT2* or *PCK2* was selectively lethal with MLH1, MSH2 and also MSH6 deficiency (Figure 5A, B & C). Confirmation of CKMT2 and PCK2 gene silencing by siRNA was performed by western blot analysis (Supplementary Figure 1B &C).

We also assessed the integrity of mitochondrial DNA in MLH1 deficient cells with PINK1 inhibition. To do this, we used quantitative PCR to measure relative levels of a gene located in mitochondrial DNA, *COX1* (aka *mt-Cot1*) (10). Silencing of PINK1 caused a significant depletion (p=0.0253) of *mt-Cot1 levels* in MLH1 deficient cells, in comparison to MLH1 proficient cells

(Supplementary Figure 3E). This suggested that the integrity of mitochondrial DNA was compromised in MLH1/PINK1 deficient cells and it is possible that the impairment of mitochondrial DNA integrity here could ultimately limit the viability of cells and explain the synthetic lethality observed.

Silencing of PINK1, CKMT2 or PCK2 leads to ROS formation

A major cause of DNA lesions such as 8-oxoG is the accumulation of reactive oxygen species (ROS) in the cell and subsequent base modification (24). Therefore to further examine the link between oxidative damage accumulation and synthetic lethality with MMR deficiency, we measured the levels of ROS in MMR deficient and proficient cells after silencing of *PINK1*, *CKMT2* or *PCK2* (Figure 5D). Increased ROS were observed upon silencing of *PINK1*, *CKMT2* and *PCK2* in all cell lines regardless of MMR status. Taken together, our data suggests that silencing of *PINK1*, *CKMT2* or *PCK2* results in an increase in ROS, ultimately leading to an accumulation of oxidative DNA damage. In MMR proficient cells, this damage is effectively repaired but in MMR deficient cells this damage remains and consequently results in cellular lethality. We also examined the levels of ROS over time upon PINK1 depletion. Silencing of PINK1 by siRNA caused an increase in ROS levels in both MSH2 deficient and proficient cells, forty-eight hours after transfection (Supplementary Figure 3F). ROS levels continued to rise until 96 hours post siRNA transfection. These observations suggested that in cells, inhibition of PINK1 leads to the generation of ROS, which can ultimately lead to the formation of 8-oxoG lesions which remain un-repaired in MMR deficient cells, resulting in cellular lethality.

***TRAP1* or *Parkin* silencing but not *HtrA2* silencing is synthetically lethal with MSH2 and MLH1 deficiency**

The PINK1 kinase phosphorylates a number of substrates including HtrA2, TRAP1 and Parkin. PINK1 phosphorylation of HtRA2 is thought to modulate the proteolytic activity of HtrA2 (25), whilst PINK1 has been reported to protect against oxidative stress by phosphorylating TRAP1 (15). PINK1-mediated phosphorylation of Parkin regulates the E3 ligase function of Parkin and it

has been suggested that Parkin function might be required for PINK1 to maintain mitochondrial homeostasis (26). Parkin expression has also been shown to be reduced in the absence of PINK1 and oxidative stress is a major determinant of morbidity in Parkin mutant flies, perhaps as a consequence of mitochondrial dysfunction (27). Therefore to further dissect the PINK1/MMR synthetic lethal interaction, we investigated whether MSH2 or MLH1 deficiency were also synthetically lethal with silencing of the PINK1 substrates HtrA2, TRAP1 and Parkin (Figure 6A-E). Interestingly, silencing of *TRAP1* or *Parkin* caused synthetic lethality in both MSH2 and MLH1 deficient models, whilst silencing of *HtrA2* did not, suggesting that the MMR/PINK1 synthetic lethalites could be mediated by loss of TRAP1 and/or Parkin function. Interestingly, Parkin deficient mice have increased ROS and decreased antioxidant activity (28) and TRAP1 is required for PINK1 mediated protection against oxidative stress-induced apoptosis (15). Of course we cannot discount the possibility that MMR/HtrA2 synthetic lethality does exist but siRNA silencing of HtrA2 was insufficient in terms of longevity or amount to elicit this effect.

Discussion

Using a number of parallel genetic screens and subsequent validation experiments, we show that MMR deficiency is synthetically lethal with targeting of the mitochondrial kinases, PINK1, CKMT2 and PCK2. In the case of PINK1, we show that silencing of the PINK1 substrates Parkin and TRAP1 also elicits MMR synthetic lethality. These synthetic lethalities are characterised by an increase in cellular ROS and also elevated levels of oxidative DNA damage. It seems reasonable to suggest that as MMR is key to the repair of oxidative lesions such as 8-oxoG (10, 16, 29, 30) the synthetic lethalities that we have observed are caused by a failure to repair oxidative DNA damage and the ultimate impairment of cellular fitness caused by these lesions. Given that MMR deficiency is a particular characteristic of some tumours, the synthetic lethalities that we have identified are worthy of further study to determine their ultimate therapeutic potential.

Previously, we had identified MMR deficiency synthetic lethalities involving DNA polymerases, using a hypothesis driven approach (10). Building from the observation that a number of DNA

polymerases were synthetically lethal with MMR gene orthologues in yeast (6, 31-33), we tested synthetic lethalities in human tumour cells and demonstrated that MSH2 deficiency was synthetically lethal with inhibition of DNA polymerase β whilst MLH1 deficiency was synthetically lethal with inhibition of DNA polymerase γ (10). It is notable that these synthetic lethalities were not "pan- MMR" effects (i.e. selective for a number of MMR deficient genotypes) but were specific for either MSH2 or MLH1 deficient genotypes, an effect we propose to be explained by the accumulation of oxidative DNA damage in either the nucleus alone (MSH2 selective) or only in mitochondrial DNA (MLH1 selective) (10). In the work presented here, we take an unbiased approach using RNAi screening and identify novel synthetic lethal interactions that are not MSH2 or MLH1 specific but indeed pan-MMR effects. Intriguingly, these pan-MMR effects are characterised by oxidative DNA damage in both the nucleus and the mitochondria, perhaps explaining their pan-MMR selective nature and consistent with our previous observations (10). In summary, this study supports our previous contention (10), which suggested that to induce MSH2 selective effects, nuclear oxidative damage is required, whilst for MLH1 selective effects mitochondrial oxidative damage is required. Here we demonstrate that to induce pan MMR effects, both nuclear and mitochondrial oxidative DNA damage is required.

The *PINK1* gene encodes a 581 amino acid putative mitochondrial serine/threonine kinase. PINK1 is encoded by the nuclear genome but, consistent with the presence of a mitochondrial targeting sequence at its N-terminus, PINK1 is localized to the mitochondria (34). PINK1 is predominately found in the mitochondrial inner membrane and intermembrane space although a fraction of PINK1 exists in the mitochondrial outer membrane with the kinase domain facing the cytosol (34-36). Over-expression of PINK1 protects cells from apoptosis in response to oxidative stress such as H_20_2 and suppresses cytochrome c relesase from the mitochondria (15). It has been suggested that PINK1 could exert this cytoprotective effects through phosphorylation of substrates such as TRAP1 (15). It has also been shown that PINK1 deficiency causes mitochondrial calcium efflux dysregulation and mitochondrial calcium overload (37). This in turn induces a rise in ROS that may further impair calcium efflux and may also inhibit glucose uptake,

resulting in reduced substrate delivery and impaired respiration. The synergistic action of increased ROS and mitochondrial calcium overload is thought to induce opening of the mitochondrial permeability transition pore, which can lead to a further increase in ROS production in the mitochondria, suggesting mechanisms by which PINK1 inhibition could ultimately cause oxidative DNA damage (38). Here we identify PINK1 as a pan- therapeutic target for MMR deficient tumours.

Our data supports the hypothesis that MMR deficiency can be targeted by causing oxidative DNA damage in both the nucleus and mitochondria, potentially highlighting the potential for using oxidative damaging agents as a therapeutic approach. Indeed, there has been much interest in therapeutically exploiting agents that cause oxidative damage as well as targeting mitochondrial proteins that lead to ROS generation (38). For example drugs such as menadione and motexafin gadolinium that are thought to elicit some of their therapeutic effects via oxidative damage, have already been used to treat certain malignancies (38). However, as with many therapeutic approaches, there is an iatrogenic risk, such that excessive oxidative damage could be pro-tumourigenic and that targeting mitochondrial proteins could cause significant normal tissue toxicity. However, it seems reasonable to think that if agents can be identified that elicit a modest level of mitochondrial and nuclear oxidative DNA damage, these could still elicit a therapeutic window in patients with MMR deficient tumours. Whilst the genes and proteins we have identified here may not be the most appropriate targets to do this (for example, mutations in the *PINK1* gene have been associated with cardiac pathologies and familial Parkinson's disease), our work has firmly established that targeting MMR deficient tumour cells by focussing on the mechanisms that modulate mitochondrial and nuclear oxidative damage is worthy of further study. For example, hypoxia is known to cause an elevation in ROS production (39), and thus combining antiangiogenic drugs that induce tumour hypoxia with other agents that cause modest oxidative DNA damage, could selectively target MMR deficient tumours, given their inherent sensitivity to the particular forms of DNA lesions caused by oxidative damage.

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FIGURE LEGENDS

FIGURE 1. **Cell viability screens with a kinase siRNA library.**

A. Illustration of a 96 well plate used in the siRNA screen, indicating the presence of positive and negative controls. For positive controls, an siRNA targeting polo-like kinase 1 (PLK1) was used and as a negative control, a non-targeting siRNA was used, siControl.

B & C. Scatter plots of delta-Z scores from cell viability screens carried out in parallel in B. HEC59 and HEC59+Chr2 cells and C. in HCT116 and HCT116+Chr3 cells. Black circles represent the differential cellular viability effect that individual siRNA SMARTpools targeting 779 kinase genes per cell line. Z scores $\leq$-2 represent significant loss of viability effects.

FIGURE 2. Validation of 20 of the most potent hits in MSH2 and MLH1 proficient and deficient cell lines

A & B. Cell viability effects of twenty of the most potent hits in **(**A) the MSH2 deficient and proficient cell lines, HEC59 and HEC59+Chr2 and (B) the MLH1 deficient and proficient cell lines, HCT116 and HCT116+Chr3. PINK1 siRNA is highlighted with a red box. Assays were performed

in triplicate. Error bars represent standard error of the mean.

FIGURE 3. Parallel RNAi screens identify PINK1 as synthetically lethal with MSH2 and MLH1.

A. Cell viability effects of *PINK1* targeting are shown in 3 MMR proficient and deficient matched pair cell lines including the MSH2 deficient and proficient cells HEC59 and HEC59+Chr2 cells, MLH1 deficient and proficient cells HCT116 and HCT116+Chr3 cells and MSH6 deficient and proficient cells, DLD1 and DLD1+Chr2 cells. PINK1 Smartpool siRNAs cause significant lethality in the MMR deficient HEC59, HCT116 and DLD1 cells and not in the MMR proficient HEC59+Chr2, HCT116+Chr3 and DLD1+Chr2 cells. * - $P \leq 0.046$ compared to the similarly transfected MMR proficient cells (Student's t-test).

B. Deficiency in MSH2 is synthetically lethal with *PINK1* inhibition. HEC59 (MSH2 deficient) and HEC59+Chr2 (MSH2 proficient) cells were transfected with either control siRNA or two different siRNA targeting *PINK1* and clonogenic assays performed. * - $P \leq 0.032$ compared to the similarly transfected MSH2 proficient HEC59+Chr2 cells (Student's t-test).

C. Deficiency in MLH1 is synthetically lethal with *PINK1* inhibition. HCT116 (MLH1 deficient) and HCT116+Chr3 (MLH1 proficient) cells were transfected with either control siRNA or two different siRNA targeting *PINK1* and clonogenic assays performed. * - $P \leq 0.023$ compared to the similarly transfected MSH2 proficient HCT116+Chr3 cells (Student's t-test).

D. Deficiency in MSH6 is synthetically lethal with *PINK1* inhibition. DLD1 (MSH6 deficient) and DLD1+Chr2 (MSH6 proficient) cells were transfected with either control siRNA or two different siRNA targeting *PINK1* and clonogenic assays performed. DLD1+Chr2. * - $P \leq 0.0466$ compared to the similarly transfected MSH2 proficient DLD1+Chr2 cells (Student's t-test).

E. HEC59+Chr2 cells were transfected with either control siRNA, PINK1 siRNA, MSH2 siRNA or in combination as indicated and clonogenic assays performed.

F. HCT116+Chr3 cells were transfected with either control siRNA, PINK1 siRNA, MLH1 siRNA or in combination as indicated and clonogenic assays performed.

FIGURE 4. Increased 8-oxoG accumulation correlates with *PINK1* inhibition and MSH2 or MLH1 deficiency

A. Increased nuclear and mitochondrial 8-oxoG accumulation upon MSH2 deficiency and silencing of *PINK1*. HEC59 and HEC59+Chr2 cells were transfected with siRNA. After 72 hrs, nuclear and mitochondrial DNA was isolated from transfected cells and analysed for 8- oxoG accumulation using an ELISA assay. Oxidised lesions were quantified according to a standard curve generated using known amounts of 8-oxoG. Assays were performed in triplicate.

B. Increased nuclear and mitochondrial 8-oxoG accumulation upon MLH1 deficiency and silencing of *PINK1*. HCT116 and HCT116+Chr3 cells were transfected with siRNA. After 72 hrs nuclear and mitochondrial DNA was isolated from transfected cells and analysed for 8-oxoG accumulation as in (A). Assays were performed in triplicate.

C. Decreased mitochondrial membrane potential upon silencing of *PINK1*. HEC59 and HEC59+Chr2 cells were transfected with siRNA. Mitochondrial membrane potential was assessed using the cyanine dye $DiIC_1(5)$. Cells were treated with either DMSO or 50⌈M CCCP as a negative and positive control for decreased mitochondrial potential.

D. Increased induction of apoptosis in HEC59 cells upon silencing of *PINK1*. Western blot of lysates from HEC59 cells, at indicated hours post transfection with either control or PINK1 siRNA. Protein lysates were immunoblotted and probed for PARP1 and β-tubulin (loading control). Arrow denotes full length and cleavage product of PARP1.

E. Increased induction of apoptosis upon MSH2 deficiency and silencing of *PINK1,* which was rescued by selenomethione treatment. Western blot of lysates from HEC59 and HEC59+Chr2 cells, at indicated hours (Hr) post transfection with PINK1 siRNA. Transfected cells were treated +/-1μM Selenomethione (Se), as indicated. Protein lysates were immunoblotted and probed for PARP1 and β-tubulin (loading control). Arrow denotes full length and cleavage product of PARP1.

FIGURE 5. Inhibition of the mitochondrial kinases, CKMT2 and PCK2 are synthetically lethal with MMR deficiency and result in increased ROS accumulation

A & B. Cell viability effects of *CKMT2* (A) *and PCK2* (B) targeting are shown in 3 MMR proficient and deficient matched pair cell lines including the MSH2 deficient and proficient cells HEC59 and HEC59+Chr2 cells, MLH1 deficient and proficient cells HCT116 and HCT116+Chr3 cells and MSH6 deficient and proficient cells, DLD1 and DLD1+Chr2 cells. CKMT2 (A) and PCK2 (B) Smartpool siRNAs cause significant lethality in the MMR deficient HEC59, HCT116 and DLD1 cells and not in the MMR proficient HEC59+Chr2, HCT116+Chr3 and DLD1+Chr2 cells. (A) * - P ≤0.0145 compared to the similarly transfected MMR proficient cells (Student's t-test). (B) * - P ≤0.003 compared to the similarly transfected MMR proficient cells (Student's t-test).

C. Deficiency in MMR is synthetically lethal with *CKMT2 and PCK2* inhibition. HEC59 (MSH2 deficient), HEC59+Chr2 (MSH2 proficient), HCT116 (MLH1 deficient), HCT116+Chr3 (MLH1 proficient), DLD1 (MSH6 deficient) and DLD1+Chr2 (MSH6 proficient) cells were transfected with either control siRNA or two different siRNA targeting either *CKMT2* or *PCK2* and cell viability was estimated five days later using CellTiter-Glo reagent.

D. Increased ROS upon silencing of *PINK1, CKMT2* and *PCK2* in HEC59, HEC59+Chr2, HCT116 and HCT116+Chr3 cells. ROS levels were analysed from transfected cells using an ELISA assay and quantified using a cell-permeable fluorogenic probe whereby the fluorescence intensity is proportional to the ROS levels. Assays were performed in triplicate.

FIGURE 6. Inhibition of the PINK1 substrates *TRAP1* and *PARKIN* but not *HtrA2*, are synthetically lethal with MMR deficiency

A**.** Deficiency in MSH2 is not synthetically lethal with *HtrA2* inhibition. HEC59 (MSH2 deficient) and HEC59+Chr2 (MSH2 proficient) cells were transfected with either control siRNA or two different siRNA targeting *HtrA2* and ATP assays performed.

B. Deficiency in MLH1 is synthetically lethal with *HtrA2* inhibition. HCT116 (MLH1 deficient) and HCT116+Chr3 (MLH1 proficient) cells were transfected with either control siRNA or two different siRNA targeting *HtrA2* and ATP assays performed.

C. Deficiency in MSH2 is synthetically lethal with *TRAP1* inhibition. HEC59 (MSH2 deficient) and HEC59+Chr2 (MSH2 proficient) cells were transfected with either control siRNA or two different

siRNA targeting *TRAP1* and ATP assays performed. * - P≤0.0143 compared to the similarly transfected MSH2 proficient HEC59+Chr2 cells (Student's t-test).

D. Deficiency in MLH1 is synthetically lethal with *TRAP1* inhibition. HCT116 (MLH1 deficient) and HCT116+Chr3 (MLH1 proficient) cells were transfected with either control siRNA or two different siRNA targeting *TRAP1* and ATP assays performed. * - P≤0.003 compared to the similarly transfected MLH1 proficient HCT116+Chr3 cells (Student's t-test).

E. Deficiency in MLH1 is synthetically lethal with *PARKIN* inhibition. HCT116 (MLH1 deficient) and HCT116+Chr3 (MLH1 proficient) cells were transfected with either control siRNA or two different siRNA targeting *PARKIN* and ATP assays performed. * - P≤0.0017 compared to the similarly transfected MLH1 proficient HCT116+Chr3 cells (Student's t-test).

TABLE 1

siRNA that were among the top scoring synthetic lethal hits in the MMR siKinome screens were a number of mitochondrial targeted genes, based on the MitoProt II predictive software (21).

MMR Synthetic Lethal Hits	**SSL with MMR Gene**	**Probable Mitochondrial Localization**	**Mitochondrial Target Seq**
C9ORF96	MLH1		YES
DKFZP434C131	MLH1		YES
GUCY2D	MLH1		YES
ICK	MLH1		YES
MAP2K6	MLH1		YES
ROR2	MLH1		YES
CKMT2	MSH2	YES	YES
FLJ23356	MSH2		
NEK11	MSH2	YES	
PCK2	MSH2	YES	YES
RPS6KB1	MSH2	YES	
LTK	MSH2		YES

Figure 1

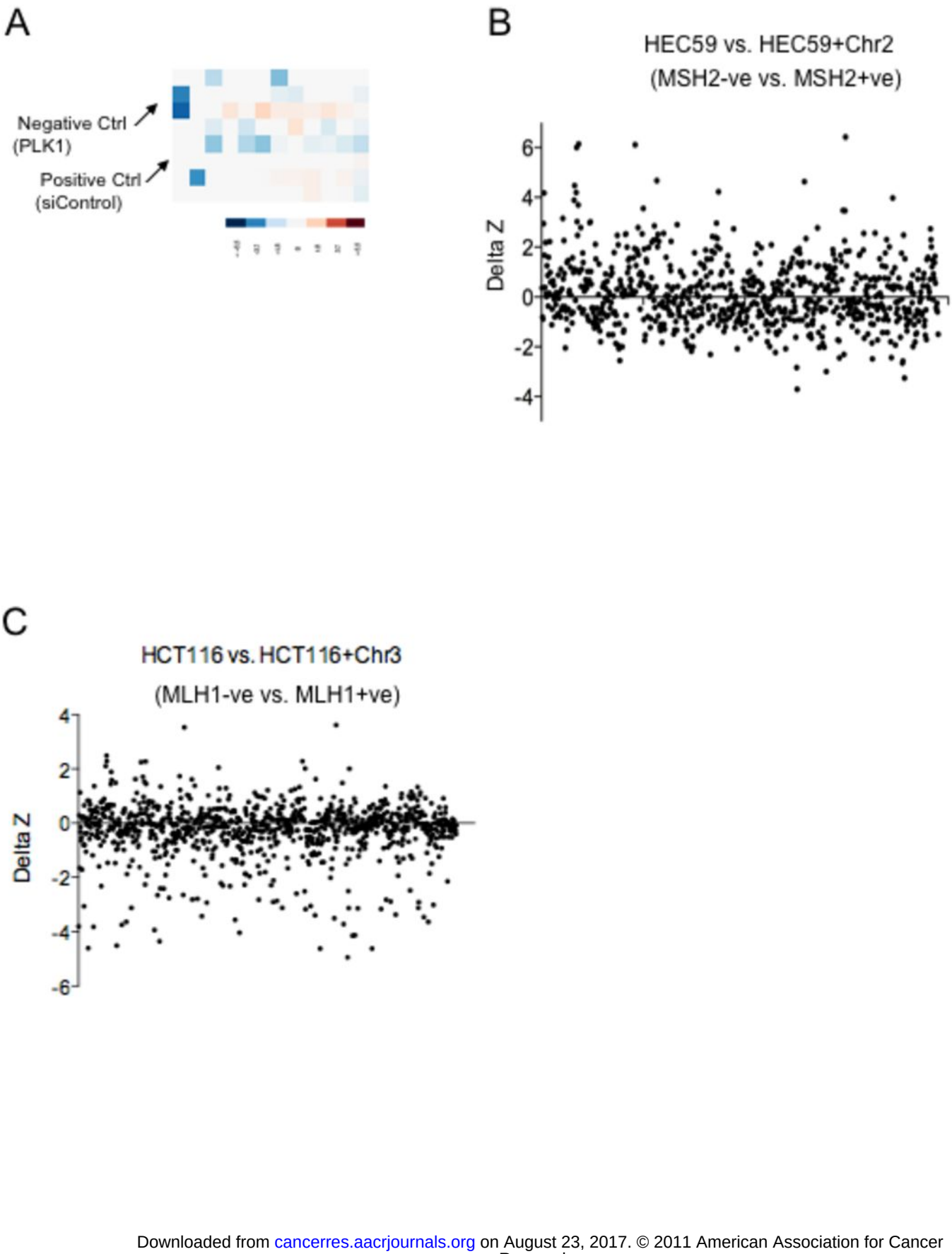
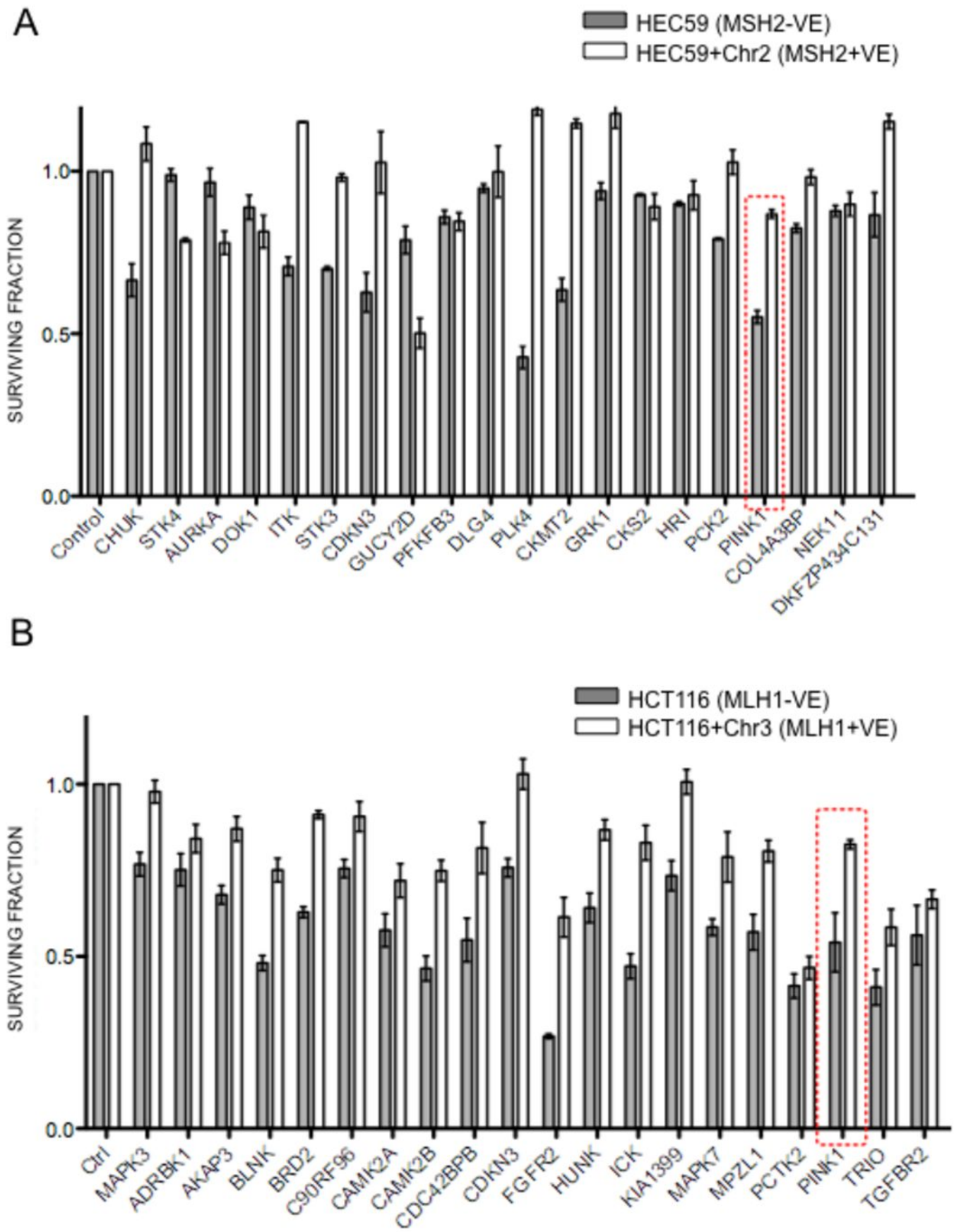


Figure 2



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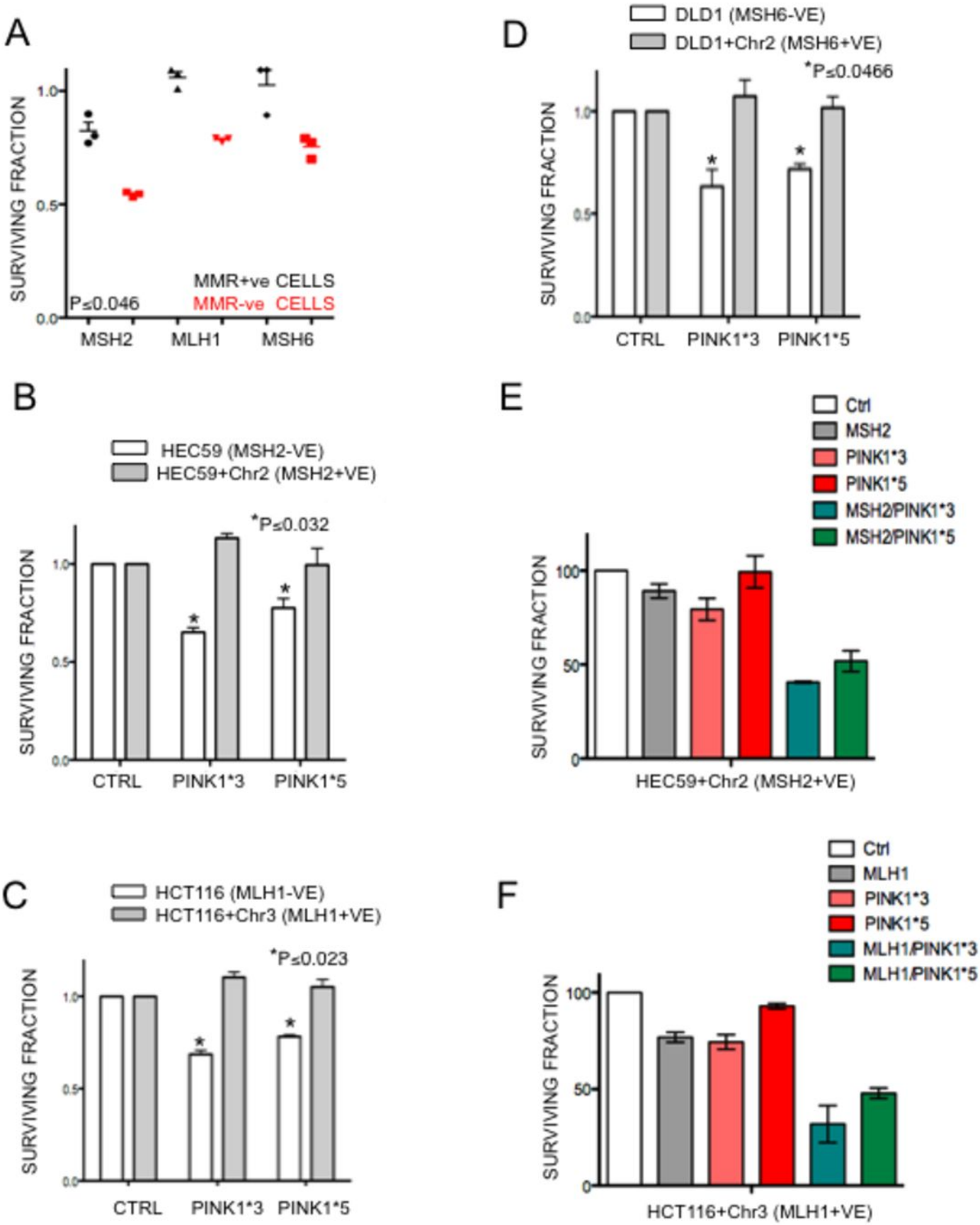
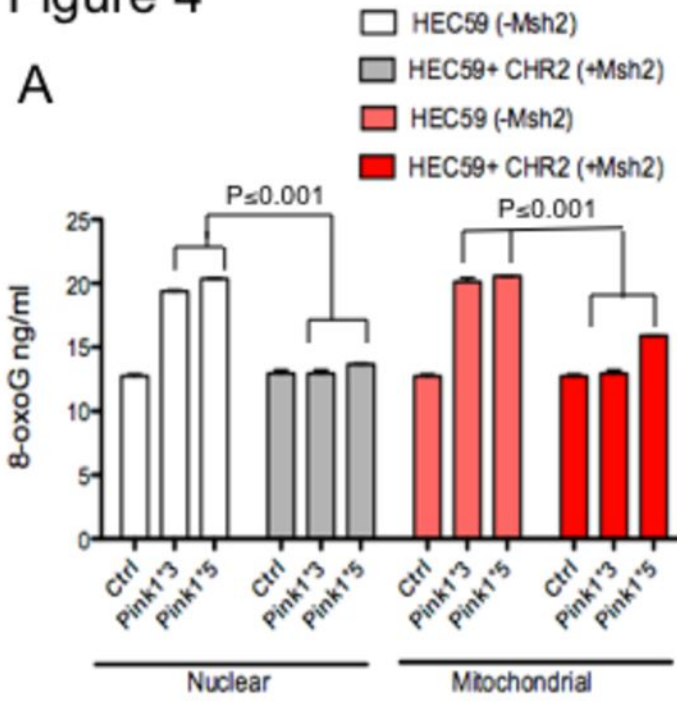
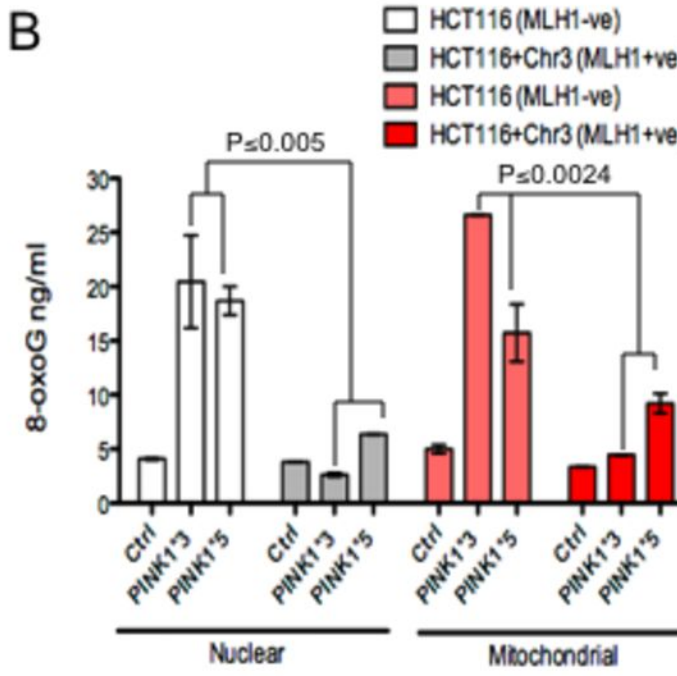
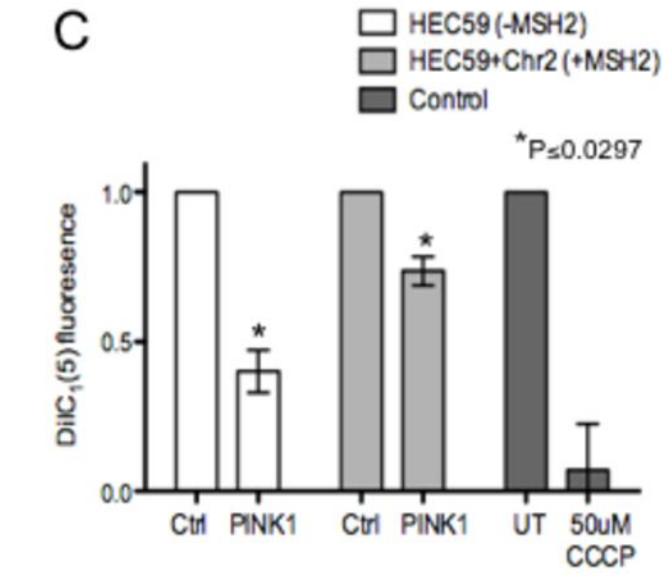
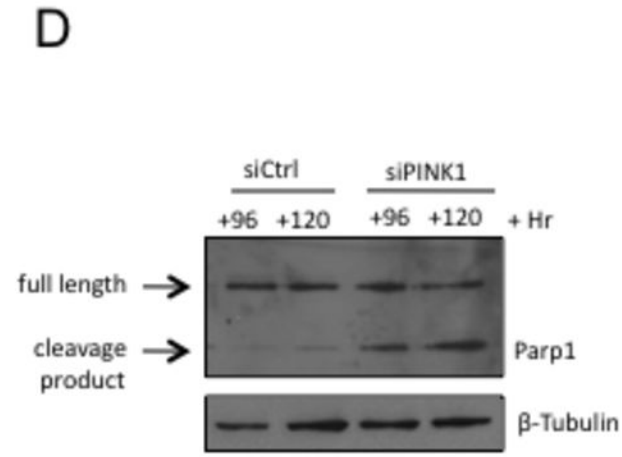


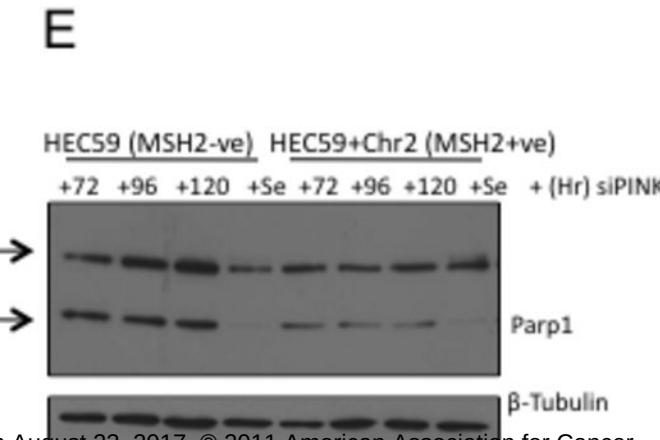
Figure 4











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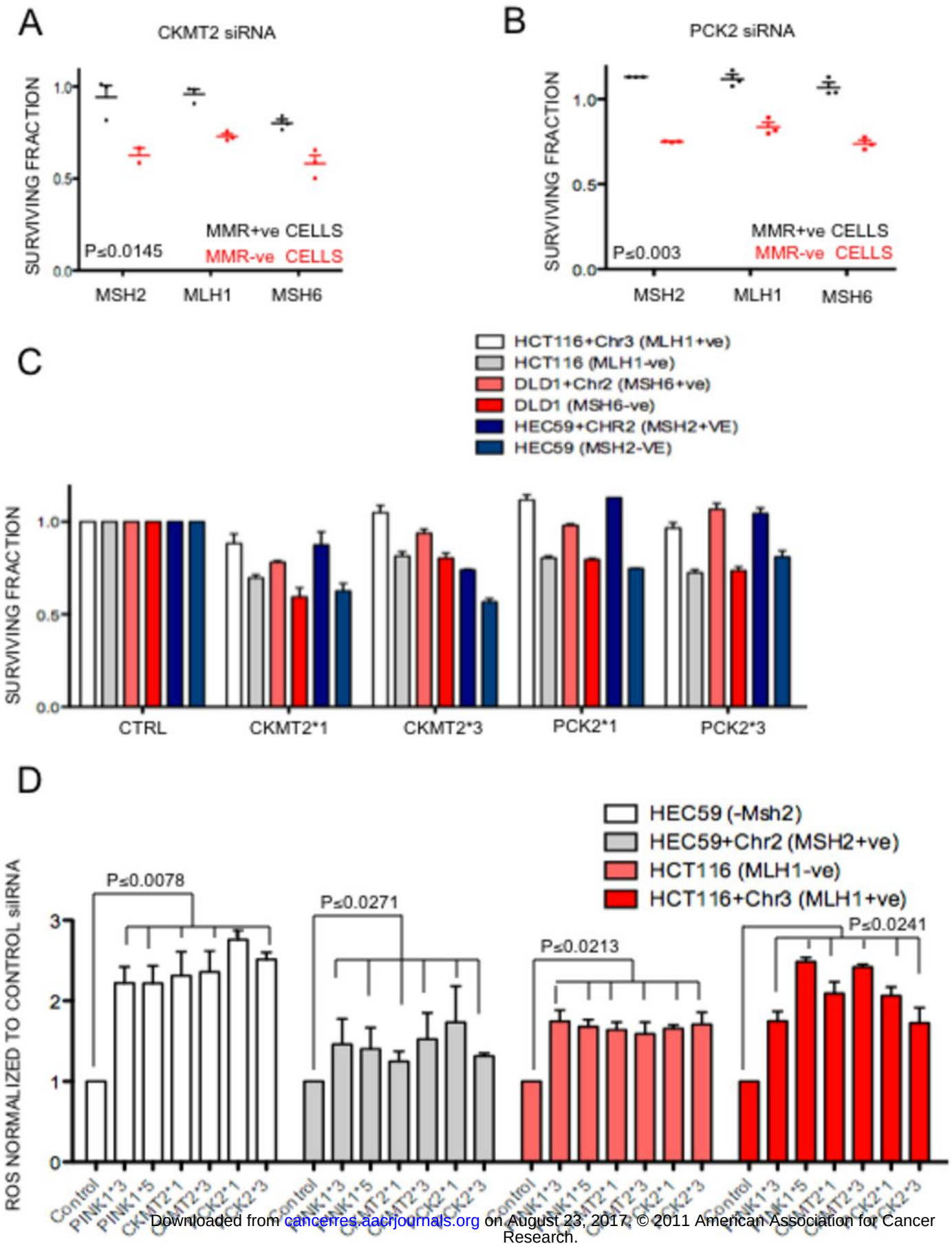

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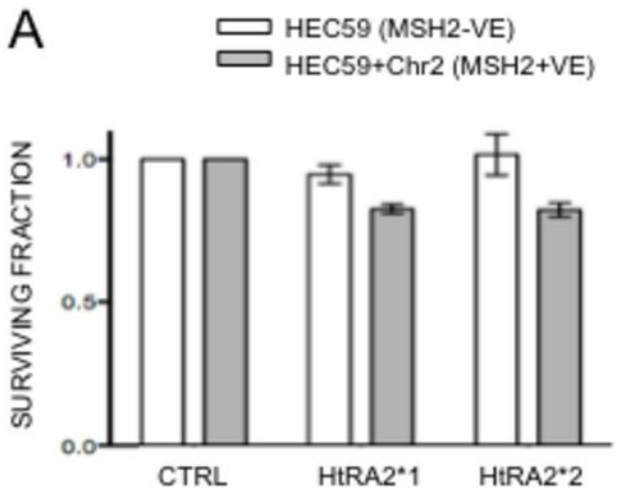

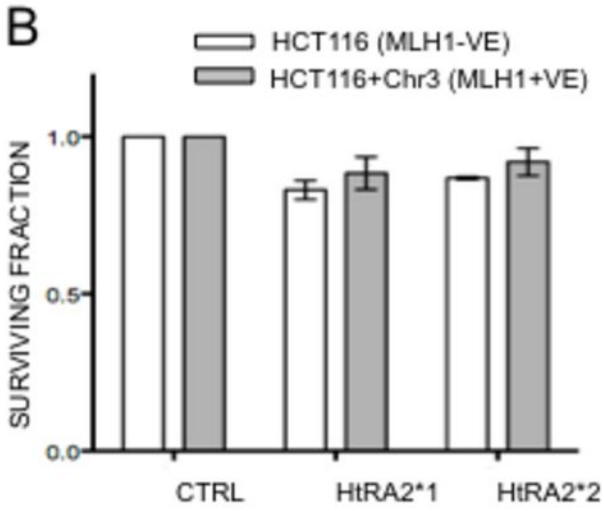

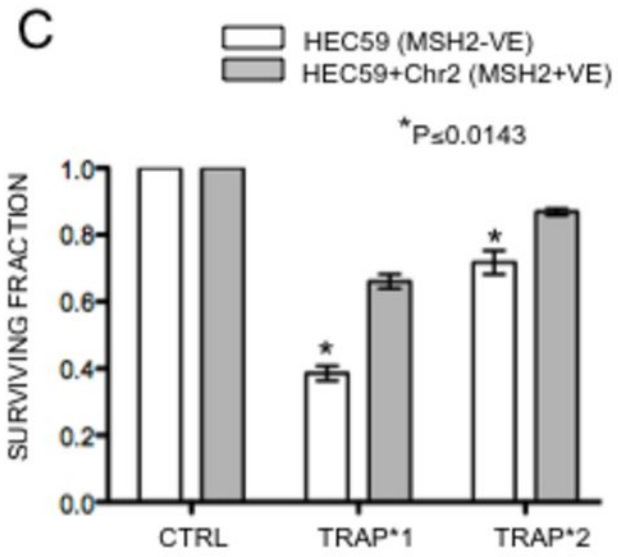

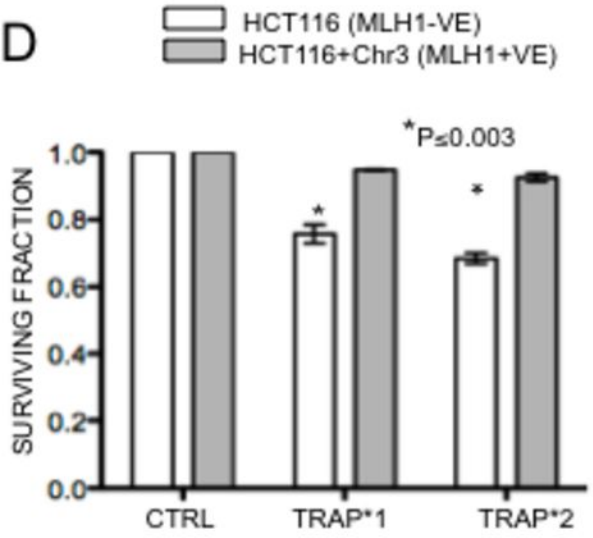

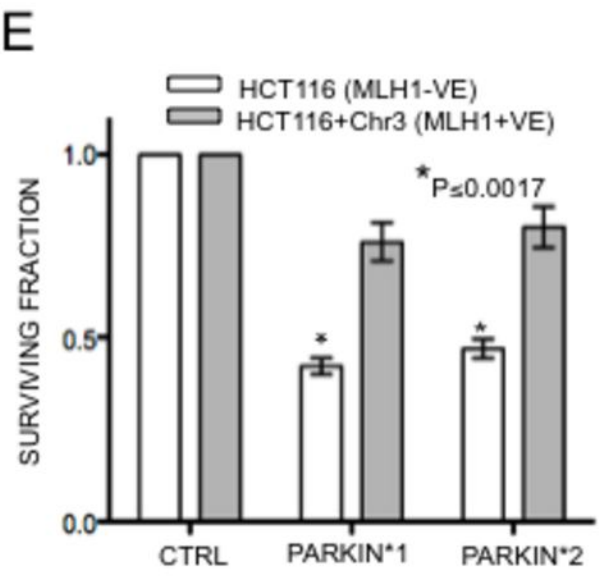

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Parallel High Throughput RNA interference Screens Identify PINK1 as a Potential Therapeutic Target for the Treatment of DNA Mismatch Repair Deficient Cancers

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