Role of CUX1 DNA Repair Function in the Resistance of Cancer Cells to Ionizing Radiation

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Abstract

More than 50% of cancer patients are treated with radiotherapy. Unfortunately, cancer cells driven by a RAS oncogene exhibit resistance to radiotherapy. Previous studies established that CUX1 knockdown is synthetic lethal to cancer cells carrying a RAS oncogene. The decrease in viability following CUX1 knockdown was associated with an increase in oxidative DNA damage. Mechanistically, the Cut repeat domains within p200 CUX1 were found to stimulate the enzymatic activities of the 8-oxoguanine DNA glycosylase 1 (OGG1) and the apurinic/apyrimidinic endonuclease 1 (APE1), two enzymes of the base excision repair (BER) pathway involved in the repair of oxidized bases and apurinic/apyrimidinic (AP) sites, respectively. In agreement with these results, repair of oxidative DNA damage is accelerated following ectopic p200 CUX1 expression and delayed after CUX1 knockdown. Since oxidized bases and apurinic/apyrimidinic (AP) sites are produced by ionizing radiation, we hypothesized that CUX1 may contribute to the resistance of cancer cells to ionizing radiation through its function as an accessory factor for OGG1 and APE1. In turn, inhibition of CUX1 DNA repair activity would sensitize cancer cells to ionizing radiation. To validate CUX1 as a therapeutic target, we initiated a series of experiments to test the effect of CUX1 knockdown and overexpression on the resistance of cancer cells to ionizing radiation. The goal of my project was to choose and optimize the assays that would enable us to perform these experiments. In parallel, I performed preliminary experiments to investigate the effects of CUX1 knockdown and overexpression on the resistance of cancer cells to ionizing radiation and other treatments that increase oxidative DNA damage. After optimizing the methyl-^{14}C thymidine incorporation assay and the single-cell gel electrophoresis (comet) assay, I observed across a panel of tumour cell lines that CUX1 knockdown sensitizes cancer cells to radiations and to an agent causing oxidative DNA damage (TH588c), whereas CUX1 overexpression confers radio-resistance. Importantly, a recombinant CUX1 protein containing only two Cut repeats (CR1CR2) is devoid of transcriptional activity, but is still able to stimulate DNA repair and confer radio-resistance. Similarly, OGG1 overexpression confers radio-resistance to DLD-1 colorectal cells, but not to "normal" retinal pigment epithelial cells (RPE1), whereas OGG1 knockdown sensitizes both DLD-1 and U251 glioblastoma cells to radiations. My work will help establish the proof-of-principle that Cut repeat domains represent a valuable therapeutic target in sensitizing tumor cells to radiotherapy.

Résumé

Plus de 50% des patients atteints de cancer sont traités par radiothérapie. Malheureusement les cellules cancéreuses stimulées par un oncogène RAS montrent une plus grande résistance à la radiothérapie. Des études antérieures ont établi que l'inhibition de l'expression du gène CUX1 cause la létalité pour les cellules cancéreuses porteuses d'un oncogène RAS, mais pas pour les cellules normales, un phénomène appelé "létalité synthétique". On a de plus montré que la létalité dans les cellules cancéreuses était associée à une augmentation des lésions oxydatives dans l'ADN. Précisément, on a trouvé que les domaines CUT (aussi appelés répétitions Cut) de l'isoforme CUX1 p200 stimulaient les activités enzymatiques de la 8-oxoguanine glycosylase 1 (OGG1) et de l'endonucléase spécifique aux sites apurinique/apyrimidinique, APE1. Ces deux enzymes exécutent les premières réactions enzymatiques de la voie de réparation des bases (BER pour Base excision repair en anglais). OGG1 a une activité glycosylique qui enlève la base oxydée, et une activité AP/lyase qui introduit une coupure simple brin. APE1 est une endonucléase qui reconnaît les sites abasiques et introduit une coupure simple brin. En accord avec ces résultats, la réparation des lésions oxydatives de l'ADN est accélérée suite à l'expression ectopique de CUX1 p200, mais retardée suite à l'inhibition de l'expression de CUX1**.** Puisque les bases nucléiques oxydées et les sites apuriniques/ apyrimidiniques représentent une large proportion des lésions produites par radiations ionisantes, nous avons émis l'hypothèse que CUX1 pouvait contribuer à la résistance des cellules cancéreuses aux radiations ionisantes par le biais de son rôle de facteur accessoire de OGG1 et APE1. À l'inverse, l'inhibition de l'activité réparatrice de CUX1 pourrait sensibiliser les cellules cancéreuses aux radiations ionisantes. La confirmation de cette hypothèse permettrait de valider CUX1 comme cible thérapeutique. Nous avons donc entrepris une série d'expériences pour tester l'effet de l'inhibition de l'expression de CUX1, ou l'effet de sa surexpression, sur la résistance des cellules cancéreuses aux radiations ionisantes. Le but de mon projet était dans un premier temps de sélectionner et d'optimiser les tests pertinents à ces expériences, et dans un deuxième temps d'effectuer des expériences préliminaires pour examiner le rôle de CUX1 dans la résistance des cellules cancéreuses aux radiations ionisantes ainsi qu'à d'autres traitements qui augmentent les lésions oxydatives dans l'ADN. Après optimisation des tests de mesure de l'incorporation de la thymidine marquée au ^{14}C et l'électrophorèse de cellules isolées (aussi appelé test de comète), j'ai pu observer dans plusieurs lignées de cellules cancéreuses que l'inhibition de l'expression de CUX1 rend les

cellules cancéreuses plus sensibles aux radiations ionisantes ainsi qu'à un agent qui produit des lésions oxydatives dans l'ADN, le TH588c. Au contraire, la surexpression de CUX1 rend les cellules cancéreuses plus résistantes à ces traitements. Plus encore, une protéine recombinante qui contient seulement deux répétitions Cut (CR1CR2) est dépourvue d'activité transcriptionnelle, mais est quand même capable de stimuler la réparation de l'ADN et de conférer la résistance aux radiations. De la même façon, la surexpression d'OGG1 confère la résistance aux radiations aux cellules colorectales DLD-1 et aux cellules de glioblastome U251. En conclusion, mes résultats contribuent à valider CUX1 comme cible thérapeutique dont l'inhibition permettrait de sensibiliser les cellules cancéreuses aux traitements de radiothérapie sans toutefois causer des effets néfastes aux cellules normales qui ne sont pas irradiées.

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Introduction

DNA Damage Responses

Our cells are continuously encountering DNA damage, experiencing an average of 10,000 oxidative damages per day[1], and are constantly working to repair and maintain genomic integrity. From DNA turnover to the mutagenic potential of alkylating and reactive oxidative species and modifying enzymes and to the mutation rate due to inaccurate DNA replication, cells experience different kinds of DNA damage to which they respond with a corresponding evolutionarily adapted DNA repair mechanism.

DNA Damage Responses (DDR) have long played an important role in cancer research, since they are of interest when tumors are found to have disrupted DNA repair mechanisms[2,3,4]. Earlier treatments targeted cancers with cytotoxic agents (radiotherapy and chemotherapy) in order to damage DNA enough to lead to cell death. More recent observations, however, indicate that certain cancer cells have developed a greater capacity than normal cells to respond to significant DNA damage. Some research efforts are directed towards identifying the proteins that are involved in DDR and are thought to be the reason for treatment resistance in cancer cells. Certain drugs that are undergoing clinical trials, such as those involving PARP1 inhibitors, aim to disrupt the efficient repair processes of cancer cells, and thereby sensitize them to DNA-damage-inducing agents.

Related research suggests that tumors may arise through defects in one kind of DDR mechanism. This would mean that cancer cells could still survive without one DDR pathway, since other DDR mechanisms could compensate for this failure, even if not as efficiently and effectively. The fact that these cancer cells lack a certain DDR pathway, however, opens up the possibility of disrupting those DDR pathways that they do employ when subjected to DNA damage.

Much of the research on disrupting those DDR pathways has centered around using siRNA screening to discover new combinations of proteins that are synthetic lethal to each other. Aside from the well-known BRCA1/2 (Homologous Recombination) and PARP-1 (SSB Repair) pair, some of these include DDB1 or XAB2 (Nucleotide Excision Repair) and the PI3K regulator PTEN[5,6]; PARP-1 and ERCC1 (Nucleotide Excision Repair)[7]; MSH2 or MLH1 (Mismatch Repair) and DNA polymerases POLβ and POLG[8]; ATM or ATR and p53[9,10]; CUX1 knockdown and RAS oncogene (Base Excision Repair)[11].

This dependency of certain cancer cells on fewer DDR pathways has led to investigation of treatments. Among others, some cases of tumor treatment resistance could occur through upregulation of DDR pathways, as suggested from a correlation between DDR upregulation in tumors and either radio- or chemo-resistance[12 13]. While the idea that tumors with upregulated DNA repair would have greater treatment resistance makes theoretical sense, these studies have not proposed the mechanism through which tumors exhibit more efficient DNA repair to resist treatment. The goal of today's research is therefore to take advantage of the weakness of those cancer cells that forego certain DNA repair pathways, with the hope that doing so would have limited impact on normal cells.

DNA Repair Pathways

Our cells have adapted to deal with a wide range of DNA damage—from mis-incorporated bases, to a set of damaged bases, single-strand breaks (SSBs) and double-stranded breaks (DSBs)—by integrating a set of DNA repair pathways, sometimes redundant, that address particular forms of DNA damage. These repair pathways have been categorized under one of three categories: direct reversal of the chemical reaction that induced the DNA damage, removal of damaged DNA bases and incorporation of new DNA, and joining of single- and double-stranded DNA breaks.

Some DNA lesions can be repaired by direct reversal of the chemical reaction that induced the DNA damage. The O6-methylguanine methyltransferase (MGMT) is able to remove the O6-MethylGuanine lesions, and the 7,8-dihydro-8-oxoguanine triphosphatase (MTH1, also known as NUDT1) can repair 8-oxo-guanine bases among others such as 8-oxoadenosine and 2-hydroxyadenosine, even before they are inserted into DNA[14].

Other DNA repair pathways involve multiple proteins and enzymatic reactions. These pathways are targeted at specific types of DNA damage and include *Nucleotide Excision Repair* (NER), *Base Excision Repair* (BER), *Mismatch Repair*, Single-Strand Break Repair (often included as part of BER) and Double-Strand Break Repair. Double-strand break repair pathways include *Homologous Recombination* (HR), *Non-Homologous End Joining* (NHEJ) and *Alternative-NHEJ*.

Cells that cannot repair DNA damage via the aforementioned repair pathways can still adapt to the damage post-replication by either using an undamaged DNA strand as template for future DNA replication, or by simply skipping the DNA damage during replication through an

error-prone polymerase. These DNA damage responses actually involve toleration of DNA damage through either *Template Switching* or *Translesion Synthesis* (Lesion Bypass), respectively[15].

Double-Strand Break Repair via Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ)

Double-stranded DNA breaks (DSBs) can either happen from single-stranded breaks on opposite DNA strands that are in close proximity to one another, or from replication of a DNA strand containing a single stranded DNA break, the latter forming what is referred to as a double-strand end. These DSBs can be caused by a wide variety of agents, from ionizing radiation, hydroyurea, alkylating agents (eg. TMZ), antimetabolites (eg. 5-FU), platinum drugs (eg. Cisplatin), and topoisomerase inhibitors (eg. Topotecan), to UV-light and X-rays. It is imperative that cells repair all DSBs, as a single one could lead to mitotic catastrophe, leading to apoptosis, through p53 activation or to necroptosis, through activation of RIPK3, or to necrosis, senescence, autophagy, pyroptosis, and caspase-independent cell death[16]. In addition, cells must repair DSBs accurately, as not doing so leads to genomic instability and possibly, cancer. Normal cells will thus undergo either HR or NHEJ to repair DSBs[14]. In both cases, the DSB is recognized by the Mre11-Rad50-NBS1 (MRN) complex, which binds to the double-stranded ends and recruits ATM. ATM phosphorylates itself as well as many downstream proteins, including BRCA1 and p53 tumor suppressors. ATM also phosphorylates histone H2AX, which recruits DNA damage response proteins to the double strand breaks. Then the cell follows either the HR or the NHEJ pathways (**Fig. 1**).

HR is the least error prone of the two DSB repair pathways, as it uses an undamaged homologous DNA strand as a template, yet it is unfortunately restricted to the late-S and G2 phases. Here the MRN complex works with CtIP to create 3'-overhangs of single stranded DNA, later aided by BLM helicase and Exo1 exonuclease activity[17]. Rad51 monomers then come together along with other proteins like BRCA1 and BRCA2 to form a large complex at the strand ends. The Rad51 complex allows for identification of a homologous DNA stretch in order to replicate the correct strand and replace the damaged one. DNA polymerases and DNA ligase I then synthesize the DNA strand, forming a Holiday junction. A pair of the following proteins GEN1/Yen1 or Slx1/Slx4 or by Mus81/Eme1, or BLM-TopIIIa complex then resolves the

junctions, the latter pair yielding non-crossover products. The other protein pairs yield both crossover and non-crossover products.

On the other hand, NHEJ is usually error-prone yet remains the predominant repair pathway in mammalian cells as it operates in all cell cycle phases. In the NHEJ pathway, the Ku70-80 heterodimer binds to the two DNA DSB ends, later recruiting DNA-PKcs to form a complex that brings the two ends together. Upon autophosphorylation, DNA-PKcs then recruit end processing enzymes like TDP1 to process the DSB ends into the ligatable 5'-P and 3'-OH termini. Polymerases later fill in the missing nucleotides, while NHEJ-specific nucleases like Artemis excise single-stranded overhangs. Similar enzymes might also include lesion-specific BER enzymes like tyrosyl-DNA phosphodiesterase 1 (TDP1), polynucleotide kinase (PNKP), apurinic/apyrimidinic endonuclease 1 (APE1), as well as exonuclease 1 (Exo1) and Werner Syndrome RecQ-Like Helicase (WRN). Importantly, recent studies suggest that Ku has an additional role of AP/deoxyribose 5'-phosphate (5'-dRP)-lyase activity, which would allow it to process abasic sites located close to the DSB in question that would otherwise prevent ligation[18] [19]. Finally, the DNA Ligase 4--X-Ray Repair Complementing Defective Repair in Chinese Hamster Cells 4—XRCC4-like factor (Lig4-XRCC4-XLF) complex ligates the DNA ends. In the absence of the Lig4-XRCC4 complex, the DNA can undergo alternative-NHEJ, which promotes chromosomal translocation[20].

Mismatch Repair

The Mismatch Repair pathway repairs bases mis-incorporated during DNA replication and/or recombination, and insertion and deletion loops overlooked by polymerases. This pathway was first discovered in *Escherichia coli* as a combination of MutS, MutH, and MutL proteins, homologous to those proteins found in humans (**Fig. 2**). In humans, briefly, a mismatch in DNA is recognized by a heterodimeric ATPase complex formed by MutS protein homolog 2 (MSH2) pairing with either MSH6 or MSH3 (**Fig. 2**). A complex with the latter preferentially recognizes large insertion/deletion loops, while the previous complex preferentially recognizes smaller loops and mismatches. Once bound to the mismatch, either complex then recruits the MutL homolog 1—PMS1 Homolog 2 (MLH1-PMS2) complex, forming a ternary complex. (An additional similar complex that has minor roles in mismatch repair is MLH1/MLH3.) The complex then instigates an incision made 3' or 5' to the lesion to allow repair done either in the 5' or 3' direction. Assembly of this ternary complex activates degradation of the strand with the

error, 5' to 3', by exonuclease 1 (Exo1). For 3'-directed excision, proteins Replication Factor C (RFC) and Proliferating Cell Nuclear Antigen (PCNA), and ATP must activate PMS2 to excise at the 5' end of the mismatch, so that Exo1 can then squeeze in and excise the mismatch in the 5' to 3' direction. Polymerase **δ** (delta) then resynthesizes the DNA, accompanied by PCNA and RPA, and DNA ligase I ligates the DNA ends together. Human MMR is thought to discriminate daughter and template strands by determining the orientation of PCNA loading on the DNA[21]. Overall, the mismatch repair system is essential in maintaining genomic integrity, as mutations in the mismatch repair pathway can lead to tumor development, evident in patients with Lynch Syndrome.

Nucleotide Excision Repair (NER)

Nucleotide Excision Repair (NER) functions in response to large DNA lesions such as bulky adducts, cyclobutane pyrimidine dimers, and 6-4 photoproducts caused by polycyclic aromatic hydrocarbons and UV light, respectively. NER can proceed in two ways: global-genome (GG) or transcription-coupled (TC) NER (**Fig. 3**). While GG-NER repairs lesions all throughout the genome, TC-NER specifically repairs lesions on DNA that is actively being transcribed. In GG-NER, the Xeroderma Pigmentosum Complementation Group C (XPC) forms a complex with UV-excision repair protein RAD23 Homolog B (HR23B) and centrin 2 (CEN2), and together they recognize the defect, along with XPE (a DNA-Damage-Binding complex with two subunits that can recognize the more subtle lesions that do not distort the helix). On the other hand, for TC-NER, damage is recognized when a DNA lesion blocks the path of an elongating RNA polymerase II (RNAPII). When this happens, proteins CSA and CSB most likely displace RNAPII to allow NER. Both TC and GG-NER then recruit TFIIH, along with two ATP-dependent helicases XPB and XPD. These latter proteins unwind the DNA strands, which (Replicating Protein A) RPA and XPA help keep separated, forming an open loop in the DNA. XPA then stimulates excision of the strand containing the lesion through recruiting XPF-ERCC1 endonuclease for the 3' incision, and XPG for the 5' incision. DNA polymerases delta, kappa, or epsilon then repair the resulting break, along with RFC, PCNA and RPA, and LIG1 or LIG3a-XRCC1 fixes the DNA backbone. Some diseases caused by NER defects include Cockayne's Syndrome (CSA and CSB defect), Xeroderma pigmentosum (XP-A to XP-G defects), and Trichothiodystrophy (XPD, XPB, and TTDA defects).

Base Excision Repair (BER)

The repair pathway most crucial to maintaining genome integrity in response to DNA damage is Base Excision Repair (BER). BER operates on a variety of base lesions, from oxidative bases, to alkylated, deaminated, and depurinated/depyrimidinated base lesions. Single-strand breaks are also repaired by BER although in this case the pathway is initiated in a different way. The most common forms of endogenous damage in cells are abasic sites (or apyrimidinic/apurinic sites—AP sites), owing in part due to being BER intermediates. Endogenous reactive oxidative species (ROS), and similarly endogenous reactive nitrogen species, create a widespread range of oxidative lesions, occurring at least 10,000 times in a cell per day[22]. The most frequent and most studied of these oxidative lesions is 8-oxoguanine (8-oxoG), occuring over 2,000 times in a cell per day[23]. Other oxidative lesions naturally occurring in DNA are ring-saturated pyrimidines such as uracil glycol, cytosine glycol, and thymine glycol. Deamination of DNA bases carrying extracyclic amino groups gives rise to lesions, most frequently cytosine and 5-methylcytosine deamination, events occurring 100-500 times in a cell per day[24 25]. According to a study done by Rydberg and Lindahl (1982), S-adenosylmethionine also can cause DNA lesions through DNA methylation, generating from 4,000 7-methylguanine, 600 3-methyladenine and 10–30 O^6-methylguanine lesions per day in a mammalian cell[26 27]. Finally, other mutated bases include, but are not limited to, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG), 4,6-diamino-5-formamidopyrimidine (FapyA), 8-oxo-adenine, 2-hydroxy-2'-deoxyadenosine (hoA), 5-hydroxymethylthymidine (hoT), 5-hydroxymethyluracil (hoU), and 5-hydroxy-2'-deoxycytosine (hoC), 1-methyladenine (1-meA), 3-methyladenine (3-meA), 3-methylcytosine (3-meC), Uracil, and Hypoxanthine (**Fig. 4**).

Briefly, in the case of altered bases, DNA glycosylases initiate BER by recognizing and excising one or more specific lesions in the DNA, resulting in an apurinic/apyrimidinic site (AP site). After being cleaved by an AP endonuclease into a single-strand break, the DNA then undergoes either short-patch or long-patch repair. Short-patch repairs one nucleotide (**Fig. 5**: bottom left panel) while long-patch repairs a string of at least two damaged nucleotides (**Fig. 5**: bottom right panel). Both pathways will involve a consequent series of enzyme activities that will lead to gap-filling, and ligation, by polymerase and ligase, respectively[28].

The aforementioned altered bases will usually have a corresponding glycosylase that will recognize it specifically. For instance, the first glycosylase discovered was uracil-DNA

glycosylase (UDG), which removes uracil from DNA. At this time, Matsumoto et al believed BER to be solely based on four enzymes: UDG, AP endonuclease (APEX1), Polymerase β, and DNA ligase (LIG3 or LIG1)[29, 30]. Later, scientists found that the enzymes used in BER depend on the state of the altered bases. For instance, 8-oxoguanine DNA glycosylase (OGG1) is used to excise 8-oxoGuanine and FapyG, the latter of which can be lethal if not immediately repaired (**Fig. 6**: right). The MutY DNA glycosylase (human) (MUTYH or MYH) excises adenine mis-paired with 8-oxo-guanine bases, among others such as 8-oxoadenosine and 2-hydroxyadenosine (**Fig. 6**: middle). The N-methylpurine-DNA glycosylase (MPG) is involved in excising alkylated damaged purines, and recognizes 3-meA and hypoxanthine[31]. Endonuclease III-like protein 1 (NTH1) and Endonuclease VIII-like 1 (NEIL1) both recognize Tg, hoU, hoC, urea, FapyG, and FapyA.

Additionally, MutT Human Homolog 1 (MTH1) has been found to reduce deoxy-triphosphase-8-oxoguanine nucleotides to deoxy-monophosphase-8-oxoguanine to prevent their incorporation in DNA. In fact, Helleday et al. recently confirmed that constitutive knockdown of MTH1 increases incorporation of 8-oxoguanine nucleotides during DNA synthesis[32] (**Fig. 6**: left). Knockdown of MTH1 decreased the viability of cancer cells, but not of nononcogenic cell lines. In fact, the Helleday group has determined that two small molecule inhibitors of MTH1, TH588c and TH287 have the same detrimental effect on cancer cells, and confirmed that these inhibitors also led to increased incorporation of 8-oxoguanine base lesions[33].

In the case of an alkylated lesion or a uracil base in the DNA, monofunctional glycosylases MPG or UDG, respectively, remove the faulty base to form an AP site. In the case of an oxidized lesion, a bifunctional DNA glycosylase—OGG1, NTH1, NEIL1, or NEIL2—forms an AP site[34]. Typically, OGG1 glycosylase is responsible for the removal of purine oxidative lesions, and NEIL1, NEIL 2, or NTH1 are responsible for the removal of pyrimidine oxidative lesions[35]. In contrast to monofunctional glycosylases, bifunctional glycosylases also have 3'AP/lyase activity that produces a single-strand break on the 3' side. This means that they do not require AP endonuclease to convert a base lesion to a strand break.

When AP sites are not repaired quickly, they block DNA replication. RNA transcription can still occur, but the AP site would cause a frameshift mutation in the mRNA which would likely lead to nonsense-mediated RNA decay. AP sites can also be converted into strand breaks or base mis-incorporations. Importantly, topoisomerase 2 is strongly attracted to free AP sites, which can lead to covalent complexes that yield fatal double strand breaks[36]. Normally, AP sites

are repaired by AP endonucleases. These make an incision into the DNA strand carrying an AP site, allowing the hydrolysis of the C3'-O-P5' bond to AP sites by nucleotidyl hydrolysases[37]. AP endonucleases attack the 5' phophodiester bonds, while AP lyases attack the 3' bonds at the AP sites[37].

Once a single-strand break has been produced, end-processing is required to generate a 3'-OH and a 5'-phosphate that can be ligated. End-processing will vary depending on which enzyme has produced the single-strand break. If the excision of the faulty base is done by a monofunctional glycosylase, either UDG or MPG, then the backbone of the DNA will be cleaved 5' to the AP site by APE1, and will result in a 3' obstructive termini with dRP[38] (**Fig. 5**: top left). In this case, Pol **β** adds a new nucleotide prior to processing the dRP. If excision is done by bifunctional glycosylases OGG1 and NTH1, obstructive termini PUA on the 5'end are formed via **β**-elimination[38]. If excision is done by bifunctional glycosylases NEIL1 and NEIL2, obstructive termini P on the 3'end are formed via **β- δ**-elimination[38]. In order to form non-obstructive termini, these recruit enzymes that will rid the DNA of the obstructive termini so as to allow base incorporation. Polymerase **β** has a dRPase that deals with the dRP termini, APE1 is recruited to 5' PUA, and PNKP is recruited to the 5' P[38]. Thus these respective enzymes can convert the obstructive termini to the normally recognized 5' OH and 3' P termini (**Fig. 5**: top panel).

Single-strand breaks can also be generated directly as a result of ROS attacking the DNA backbone. Attack at the sugar residue leads to fragmentation, base loss, and strand breaks with a terminal sugar residue fragment[39 40 41]. These SSBs are rapidly recognized by PARP1. Binding of PARP1 to SSBs is rapidly followed by poly-ADP-ribosylation of PARP1 itself, which helps recruit enzymes that can process the DNA ends to produce the correct 3'-OH and 5'-phosphate ends: TDP1 (tyrosyl-DNA phosphodiesterase 1), APTX (aprataxin), PNK (polynucleotide kinase), APE1 (apurinic/apyrimidinic endonuclease 1)[42].

Repair synthesis can proceed via either short-patch or long-patch pathways. Short-patch repair pathway involves addition of one new nucleotide, while long-patch involves the addition of two or more nucleotides. Short-patch BER/SSBR involves Polymerase **β** aided by the XRCC1 scaffold, followed by LIG3a ligating the DNA[38]. Long-patch BER/SSBR involves Polymerase **β** and/or Polymerase **δ**/**ε**, aided by PCNA and RFC. The resulting string of nucleotides there prior to the polymerase activity is removed by FEN1, aided by PCNA[38]. LIG1, aided by PCNA, ligates the new bases to the DNA[38].

In contrast to other DNA repair pathways, with the exception of *MYH*, there is no inherited mutation that inactivates BER. It is assumed that BER is absolutely essential and therefore that BER inactivating mutations are embryonic lethal, as suggested from the phenotypes of several knockout mice[43]. In order to determine the function of these proteins in BER, scientists measured the differences in DNA damage response with regard to the expression of various BER proteins. Associations studied between BER proteins and diseases are few but interesting. Inherited missense mutations within *MYH*, a DNA glycosylase that catalyzes the excision of A from oxoG·A mispairs, reduce the capacity to repair oxoG·A mismatches, leading to increased numbers of G-to-T transversions[44 45]. As a consequence, it is estimated that the sheer number of adenomatous polyps in individuals with MYH mutations leads to an almost 100% lifetime risk of eventually developing a carcinoma[46]. Interestingly, two recent studies on OGG1 and NEIL1 knockout mice showed that glycosylase knockdown actually **decreased** the risk for Huntington's disease[47 48]. This observation is based on the link of OGG1 and NEIL1 base processing in trinucleotide repeats of the CAG type to the trinucleotide expansion seen in Huntington's disease. So while BER remains an essential DNA repair pathway, in a particular genetic background at a specific sequence, too much glycosylase activity could be harmful. Speaking of neurological disorders, a review suggests that 6 neurological disorders (spinocerebellar ataxia with axonal neuropathy-1, Huntington's disease, Alzheimer's disease, Parkinson's disease, Down syndrome and amyotrophic lateral sclerosis) result from the inability of BER to deal with increasing oxidative stress, but it is unclear if the increase in oxidative stress is a result of impaired BER, or if there is something else increasing oxidative stress to the point that BER is not enough to repair the resulting damage[49]. Clearly, there are still many enticing mysteries surrounding the field of BER waiting to be solved.

Standard of Care Cancer Treatments

Surgery

The oldest and most reliable and effective treatment against cancer is surgery. In fact, in 1750 BCE, the Babylonian code of Hammurabi listed standard fees for the surgical removal of tumors (10 shekels) and penalties for failure[50]. In the case of surgery, different procedures are required depending on whether a lesion is benign or malignant. Benign lesions are growths that occur when cells divide abnormally but are contained within an area. Malignant lesions will

invade nearby tissues and metastasize to other areas of the body. If imaging (eg. CT scan, MRI, X-ray, and Ultrasound) procedures cannot confirm that a lesion is benign, then a biopsy is done and sent to a lab for testing[52].

Surgery also tells clinicians how to stage cancer. During surgical biopsies, when needle biopsies are insufficient, surgeons and pathologists examine the area around the lesion, including the lymph nodes and nearby organs. Depending on the level of invasion of the lesion, surgeons will ascribe the following staging grades: T 1-4 to denote primary tumor size, N 0-3 to denote regional lymph node metastasis, M 0-1 to denote distant metastasis[52]. This staging is important to ascertain the following course of treatment to give to patients.

Surgery is not essential in the case of benign tumors, unless they are causing symptoms in patients. Surgery can also be used to remove non-vital and non-diseased organs to prevent cancer in at-high-risk patients. For example, Angelina Jolie made headlines when she underwent a prophylactic mastectomy on both breasts because she was carrying a mutation within the BRCA1 tumor suppressor gene, and as such had a high risk of developing breast cancer.

If a malignant tumor is detected early enough that it is contained within an organ, the patient can undergo curative surgery to remove part of or the whole affected organ[51]. Curative surgery can also be used in conjunction with radio- or chemo-therapy. When the tumor load is in one area but is too big to fully remove, surgery is used to take away as much tumor as possible, and the rest of the tumor is treated with either radio- and/or chemo-therapy, as is done in some cases of lymphoma and ovarian cancer[51].

Unfortunately, if the disease progresses too much, surgery is only used for palliative purposes to alleviate the discomfort of the patient. Importantly, surgical oncology is not completely useless in metastasis. Aside from its usefulness in palliative treatment, it is also effective in some patients with solitary metastases in the lung, such as is seen in some patients with metastatic sarcoma[52]. While these cases are rare, they should remain considered before out-ruling surgery in a patient presenting with metastatic disease[52].

Radiotherapy

Ionizing Radiation (IR) is commonly used in treatments for cancer, both as curative and palliative treatment, on the principle that it instigates enough DNA damage in cells to cause cell apoptosis, necrosis, or senescence. As this would induce the death of normal cells as well, irradiation is targeted at the specific tumorous area. Additionally, in the clinic, for curative

radiotherapy, a large dose of irradiation will be fractionated, and delivered generally in multiple fractions of 2 Gy, over a length of time[53]. In theory doing so will allow normal cells to repair irradiation-induced damage while still killing cancer cells. Theoretically, cancer cells are more affected than normal cells, as they exhibit greater metabolic activity and presumably divide faster than normal cells. In addition, certain cancer cells produce higher amounts of reactive oxidative species (ROS) than normal cells. In turn, cancer cells may express higher levels of DNA repair proteins to cope with this increase in ROS and oxidative DNA damage. Ideally, IR would cause a greater accumulation of ROS in cancer cells, surpassing the cells' tolerance of DNA damage[54].

DNA direct absorption of IR can often lead to double strand breaks. As seen earlier, these breaks are repaired either by the NHEJ or HR pathway. Since NHEJ is error-prone, survival of tumor cells rely on efficient HR thus encouraging development of therapies that target this pathway[55]. For example, cancer cells with defects in the HR pathway will be more sensitive to radiation treatment, and even more so to a drug given in combination with radiotherapy that inhibits a protein in another DNA repair pathway.

In addition, IR produces Reactive Oxidative Species (ROS). ROS can create many different types of damage to cellular components, from lipid peroxidation in cell membranes to oxidative lesions in DNA[56]. Irradiation typically creates ROS by hydrolyzing water, creating hydroxyl radicals[57]. These radicals bind very strongly to DNA, attacking the double bonds of DNA bases and removing hydrogen ions from either the sugar phosphate backbone (creating strand breaks or strand modifications) or from the methyl group on thymine or from the C-H bonds of 2'-deoxyribose (the latter two creating oxidative lesions)[58]. The altered bases are excised, resulting in abasic sites that could lead to single strand breaks. Even without irradiation, there are endogenous ROS—present in small quantities due to metabolism—but that could result only in single strand breaks, as the probability of two lesions occurring in close proximity is low[59]. However, IR generates high local concentration of ROS near DNA, which leads to clusters of DNA lesions grouped closer together. These lesions would be recognized by BER and converted to an AP site (BER intermediate), which could be converted to single-strand breaks. Since single-strand breaks in close proximity to each other give rise to a double-strand break, IR would yield greater amounts of lethal DNA double-strand breaks through increasing secondary double-strand breaks created through BER intermediates (**Fig. 7A**).

ROS causes a wide range of damage in DNA. The most common resulting damaged base is 8-hydroxyguanine. As detailed earlier, 8-oxoguanine residues are recognized and removed

primarily by the OGG1 DNA glycosylase. If not repaired prior to DNA replication, 8-oxoguanine can lead to misincorporation of adenine on the opposite strand. This mispairing is solved by MYH that excises the newly formed adenine, distinguishing the adenine as the mispaired base, and not the guanine, by singling out the newly replicated strand as the one with least modifications. This correction provides another opportunity for OGG1 to remove the 8-oxoguanine and initiate base excision repair (BER) (**Fig. 6**).

Radiation can also be used in combination with surgery and chemotherapy. Radiotherapy prior to tumor resection can decrease the tumor burden and render an unresectable tumor resectable[52]. Radiotherapy can additionally be used after surgery if metastases are found during surgery, but also if the tumor burden is too large to be completely removed surgically. Similarly, radiotherapy can also be used in combination with chemotherapy to target certain areas, whereas chemotherapy would usually affect the whole body. Systemic chemotherapy can also be used in combination with regional radiotherapy to sensitive cancer cells to radiotherapy, depending on the agents chosen[52].

Chemotherapy

Chemotherapy employs drugs that are given to the patient either orally or through transfusions. Chemotherapy can be used either as a primary treatment for patients with late and measureable disease, or in combination with radiotherapy or surgery, either prior to or after surgery to limit micrometastatic disease, referred to as neo-adjuvant and adjuvant chemotherapy, respectively. Since most standard single drugs have been unable to cure cancer (apart from methotrexate used to treat choriocarcinoma, a malignant uterine tumor, and cyclophosphamide used to treat Burkitt's lymphoma[52]), chemotherapeutic treatment usually involves a combination of drugs that target different characteristics of cancer cells with the hope that doing so will eliminate cancer cells that might have held or acquired resistance to one drug. The complexity of the chemotherapeutic regimen is manifested in the list of drugs present in a typical cancer patient's medical history. Most treatments will incorporate a combination of drugs[52]. An example of a chemotherapeutic regimen might include an alkylating agent, an anti-microtubule agent and either a topoisomerase inhibitor or an antimetabolite. All agents either interfere with DNA replication (antimetabolites), cause DNA damage (alkylating agents, topoisomerase inhibitors) or impede proper chromosome segregation (microtubule inhibitors). In addition, some drugs

interfere with the repair of DNA lesions that arise spontaneously or are caused by treatments (PARP1 inhibitors). The combination of treatments actually allows for greater cell kill, as well as targets a wider variety of tumor cells, addressing the problem of tumor heterogeneity and drug resistance. The combination of multiple drugs may or may not necessarily increase the therapeutic window, since each drug treatment is associated with a number of side-effects.

As an application of the synthetic lethality concept, some newly approved drugs aim to take advantage of mutations in cancer cells. For example, PARP-1 inhibitors are used against BRCA mutant-carrying cancer cells. PARP1 inhibition will prevent the repair of single-strand breaks (SSBs). During DNA replication, unrepaired SSBs are converted to a type of DSB called double-strand end. These lesions can only be repaired by a process involving homology-dependent repair. Since BRCA-defective cancer cells are unable to perform homologous recombination, they are much more sensitive to a PARP-1 inhibitor, than are normal cells[60]. One problem with PARP-1 inhibitors is that they also inhibit PARP2. While Parp1 and Parp2 knockout mice are viable, double knockout mice are embryonic lethal, clearly showing that PARP1/2 are essential[61]. Indeed, PARP1 inhibitors can cause serious adverse effects that limit their use.

Some chemotherapeutic agents fall under the category of microtubule (or mitotic) inhibitors. These inhibit microtubules that are responsible for pulling apart the chromosomes during cell division. Since cancer cells are believed to proliferate faster than normal cells, they would be most affected by microtubule inhibitors that would block their cell division. Microtubule inhibitors either inhibit polymerization or depolymerization of microtubules. Taxanes and epothilones stabilize microtubules thereby inhibiting depolymerization, and include ixabepilone, doxecatel, and paclitaxel[62]. On the other hand, vinca alkaloids and estramustine inhibit microtubule polymerization and include vinblastine, vincristine, and vinorelbine[62]. A disadvantage to this treatment is that it can cause nerve damage, which significantly limits the amount that can be given[52].

Alkylating drugs are among the most common chemotherapeutic drugs that inflict damage on DNA of cancer cells. These drugs can cause mono-alkylation adducts and interstrand or intrastrand cross-linking depending on the drug. Monofunctional alkylating agents cause lesions in one of the two DNA strands through base or phosphate modifications. Some of these drugs include procarbazine and dacarbazine (temozolomide is a derivative of dacarbazine) [63] and cause mono-alkylation adducts. Other drugs can form interstrand and intrastrand crosslinks,

either covalently linking bases on opposite strands or within the same strand of DNA, respectively. Bifunctional alkylating agents such as nitrosureas class drugs (eg. lomustine and carmustine), nitrogen mustards (eg. cyclophosphamide, melphalan, and chlorambucil), and alkylating neoplastic agents (eg. altretamine and busulfan) cause interstrand crosslinking, while platinum-based drugs (eg. cisplatin, oxalaplatin, and carboplatin)[64] cause intrastrand crosslinking. Temozolomide in particular is commonly used in glioblastoma multiforme in combination with surgery and radiotherapy. Its mechanism of action lies in methylating DNA purine bases (O6-guanine; N7-guanine and N3-adenine)[65]. MGMT is able to remove the O6-MeG lesions, Base Excision Repair proteins can remove the other two lesions, and the damage is tolerated in cancer cells that are deficient in mismatch repair[65]. Thus targeting DNA repair proteins is a current strategy in overcoming resistance to TMZ, from inhibiting MGMT, to trials inhibiting PARP-1 and APE-1, BER proteins[65]. The disadvantage to using alkylating agents is that it also inflicts DNA damage on normal cells, especially in the bone marrow, with associated toxicities[52].

Some drugs, such as bleomycin, an anti-tumor antibiotic, are able to cleave the sugar phosphate backbone of DNA, causing either single- or double-stranded DNA breaks[66]. Other drugs that fall under the category of anti-tumor antibiotics actually interfere with DNA replication enzymes, such as the anthracyclines daunorubicin, doxorubicin, epirubicin, and idarubicin, all of which are topoisomerase inhibitors. Similar to bleomycin, anti-tumor antibiotics that are not anthracyclines include actinomycin-D, mitomycin-D, and mitoxantrone (also a topoisomerase inhibitor). These drugs are very toxic, and as such given in very limited doses to the patient, to limit heart and lung disease.

Other chemotherapeutic drugs are indirect modulators of the DNA damage response, either inhibiting DNA synthesis proteins like DNA polymerase, or epigenetic regulators like DNA methyltransferase 1 (DNMT1), or even DNA replication proteins, like topoisomerases. Topoisomerases play an important role in DNA replication as they prevent tangling of DNA during replication. Topoisomerase 1 causes a single-strand break in DNA allowing the DNA at either side to twist in a way that releases tension[67]. In the event of two double helices crossing over each other, topoisomerase 2 creates a transient double-strand break in one double helix, allowing the other to pass through[67]. In reference to the latter case, topoisomerase 1 (TOP1) has been a target for inhibitors in combinational chemotherapy. For example, inhibitors irinotecan and topotecan are used in first-line treatment of colorectal cancers in combination with 5-

fluorouracil, and as second-line treatment for ovary, cervix and small-cell lung cancers in combination with cisplatin[68]. Topoisomerase II inhibitors include etoposide, teniposide, and mitoxantrone (also an anti-tumor antibiotic) and anthracyclines, but these are less used as they have recently been shown to cause secondary cancers (most often acute myelogenous leukemia, or AML) as rapidly as 2 to 3 years after treatment.

Finally, antimetabolites are also commonly used in cancer therapy as they block DNA and RNA synthesis by introducing themselves as pseudo-nucleotides or pseudo-bases, and thus halting cell growth and division[52]. They are most prone to inflict damage on dividing cells, and as such are apparently more selective for cancer cells. These drugs include 5-fluorouracil, 6-mercaptopurine, capecitabine, floxuridine, fludarabine, gemcitabine, hydroxyurea, methotrexate, and pemetrexed. Side effects include bleeding and sensitivity to infections[52].

Resistance to Treatment

The major cause of death in cancer patients lies in cancer resistance to treatment. In many cases, the treatment will affect the tumor initially, and kill many cancer cells, yet leaving a few cancer cells remaining. These cancer cells are either in a place that the drug could not reach (for example, they could have escaped behind the blood-brain barrier) or they had upregulated drug pumps, like p-glycoprotein, that expulsed the drug from the cell and prevented it from reaching its target (although no pumps were found that corresponded to alkylating agents[69]), or they entered a quiescent cell state, characteristic of cancer stem cells[70]. According to Borst, these cancer stem cells have elevated levels of drug transporters, and exhibit resistance to DNA damage—especially to IR, due to increased capacity for DNA repair or to the quiescent-like state of the cells—quiescence[70]. Of interest, many of these properties are associated with the epithelial to mesenchymal transition.

Whether cancer resistance to treatment is due to a reversible quiescent phase or to more efficient DNA repair is unknown. The possibility remains that while DNA repair defects promote tumorigenesis, these same defects would be useless in a mature tumor. These DNA repair defects could be reversed, whether done before or after treatment, to allow some cancer cells to adapt to the overwhelming DNA damage caused by radiotherapy and chemotherapy. Thus the cancer cells could increase their capacity to repair oxidative DNA damage through increasing the expression of certain DNA repair proteins involved in repair of oxidative DNA damage, as suggested by the correlation between overexpression of DNA repair genes and metastatic

potential in cancer cells seen in studies reviewed by Alain Sarasin et al[71]. Recently there have been ongoing clinical trials of treatments targeting certain proteins involved in DNA repair, but unfortunately many have a small therapeutic window and induce harmful side effects, as they are essential or relied upon by normal cells. An ideal target could be a protein overexpressed in cancer cells that is required for the survival of cancer cells but is not needed, or less so, in normal cells. Involved in DNA repair, this protein would function as an accessory factor that aids DNA repair activity through stimulation of DNA repair protein, while not having an effect on its own. Inhibiting such a target would in theory not have an effect in normal cells. Common experiments that are used to investigate the best treatment for patients involve clonogenic, thymidine incorporation, and DiSC assays performed on a sampling of a patient's tumor, whether they exhibit advanced or early stage disease.

Ongoing Clinical Trials

In addition to drugs aforementioned exploiting the cancer cell's excessive proliferation (i.e. microtubule inhibitors) and relatively disrupted DNA repair (ie. alkylating agents), there are a number of compounds that directly target the DDR pathways. As Pearl et al. mention in their review, there are several direct DDR inhibitors undergoing clinical investigation: BER proteins APE-1 (apurinic/apyrimidinic endonuclease 1) and PARP-1, direct repair protein MGMT (6-O-methylguanine DNA methyltransferase), NHEJ proteins DNA-PKcs (DNA-dependent protein kinase), telomere maintenance protein TERT (telomerase reverse transcriptase), HR protein ATR, topoisomerase inhibitors TOPO I and II, and cell cycle checkpoint inhibitors ATM, ATR, CHCK 1, CHCK 2, and WEE1[68].

A particularly exciting clinical trial is one that involves Base Excision Repair protein APE1. An APE1 inhibitor recently successfully passed phase 1 clinical trial with good results[72]. TRC102 is an APE1 inhibitor as it binds to AP sites generated by base excision repair, and instigates topoisomerase II-dependent double strand breaks and apoptosis[72]. This is supposedly selective for cancer cells since certain cancer cells overexpress topoisomerase II. In normal cells topoisomerase II is removed and the AP site is repaired by the long patch base excision repair. A phase 2 study is underway, but it is nonetheless a promising study that will hopefully pave the way for more clinical trials focused on inhibiting the base excision repair pathway in cancer cells. This study gives Dr. Nepveu's lab validation for studying CUX1's role in the BER pathway in cancer cells, as his lab has found it stimulates BER enzymes OGG1 and APE1.

Harmful Side Effects

With regards to choosing chemotherapeutic regimes for their patients, clinicians pay close attention to the side effects that are associated with the compounds explored in clinical trials. While scientists might rejoice at seeing complete regression of a tumor from a potential new compound, they must keep in mind that the compound will be a complete failure and will be completely rejected by the clinician if it were to cause too serious adverse effects, such as brain damage, and significantly affect quality of life of the patient. Too often is the bench far removed from the bedside of the patient.

As of now, in some of the aforementioned clinical trials with base excision repair proteins, a challenge has been overcoming certain adverse effects. In trials involving PARP-1 inhibitors, for instance, there were many cases of lower white blood cell counts in patients after being given the compound in question[73 74 75 76]. Other alarming effects include, but are not limited to, pyrexia, anemia, nausea/vomiting, fatigue, dyspnea (breathing difficulty), dyspepsia (indigestion), asthenia (loss of body strength), myalgia (muscle pain), alopecia, peripheral oedema, deep vein thrombosis and arthralgia (joint pain)[73 74 75 76]. While most of these side effects do not appear immediately life threatening, they can lead to cachexia in patients, presenting through a decreased will to eat and exercise. Cachexia, a muscle-wasting syndrome, greatly weakens the cancer patient, and is ultimately the cause of death in many cancer patients. These side effects, we believe, could be avoided if we were to target instead a protein that was relied upon by cancer cells to promote DNA repair, and that was simultaneously non-essential in normal cells.

***In Vitro* Testing of Chemo- and Radio-Sensitivity of Tumors**

A wide range of experiments are performed to determine sensitivity of certain tumor cell lines to chemo and radio-therapy, including the clonogenic assay, the thymidine incorporation assay, and treatment of mice with human tumor xenografts. Importantly, when comparing the results of these *in vitro* assays to patient response to the same treatments, Fruehauf generally found a strong correlation, with an overall sensitivity of 85% and an overall specificity of 80%[77]. As these assays measure different things, it can be useful to perform several of these assays with the same tumor sample, to confirm tumor chemosensitivity. Specifically, clonogenic assays measure colony growth and formation from single cells, thymidine incorporation assays measure rate of DNA synthesis (ie. proliferative capacity) of cell population, and other assays like MTT

can measure mitochondrial enzyme activity or metabolic activity. Compared to the clonogenic assay, the thymidine incorporation assay is faster, yielding results in 3-5 days, as opposed to the 10-18 days necessary for the clonogenic assay. Another assay that can be used (not used in this thesis) is the DiSC (Differential Staining Cytotoxicity) assay, which measures amount of dead cancer cells against live cells, differentiating between dead malignant cells and contaminant nonmalignant cells through staining[77 78]. Overall these different methods can provide clinicians and scientists with a general idea of what chemotherapeutic agents or drug candidates would work most effectively in killing cancer cells. In this thesis, I have performed both clonogenic and thymidine incorporation assays to measure tumor response to radiation and knockdown of CUX1, an accessory factor to DNA repair that is needed in some cancer cells but is not essential in normal cells.

CUX1, Haploinsufficient Tumor Suppressor Gene

Cut Homeobox 1, CUX1, has been marked as a haploinsufficient tumor suppressor gene on 7q22.1[79] (reviewed in [80]). Loss-of-heterozygosity (LOH) affecting the 7q22 region occurs in approximately 18% of breast cancers[81], and up to 40-50% in secondary leukemias[82]. In addition, inactivating point mutations have been reported in 1-5% of cancers in which the two CUX1 alleles are present[83]. Paradoxically, CUX1 gene copy number is increased in ~71% of human cancers (Sanger cell line website). Moreover, in many cancers CUX1 expression inversely correlates with patient survival. This inverse correlation was first established from immunohistochemical studies in breast and pancreatic cancers[84 85 86]. Unfortunately, expression profiling using microarrays do not provide relevant information because the oligos are taken from a region more than 25 Kbp downstream of CUX1 and contain exons for the Cut alternatively spliced product, CASP, a protein that localizes to the golgi (reviewed in [80]). RNA sequencing revealed a strong inverse correlation between CUX1 expression and patient survival in glioma, glioblastomas and colorectal cancers[87]. Using a score that combines gene copy number and gene expression, a TCGA study ranked CUX1 as the fifth gene associated with tumor progression[86]. The challenge has been to identify the biochemical and cellular functions of CUX1 that protect against cancer and those that promote cancer progression.

CUX1 codes for many protein isoforms with distinct DNA binding and transcriptional properties, named after their apparent molecular weight, and include p200, p110, p75 CUX1 isoforms. The full-length protein, p200 CUX1, contains four evolutionarily conserved DNA-

binding domains: three CUT repeats (CR1, CR2, and CR3) and a CUT homeodomain (HD) (**Fig. 7B**). The p200 CUX1 protein binds DNA transiently, and in mid-G1 phase 1 to 10% of p200 CUX1 is proteolytically processed by a nuclear isoform of cathepsin L to produce the p110 isoform[88 89 90 91].

In the past few years, Dr. Nepveu's lab made breaking discoveries concerning CUX1's role in DNA repair. Although they had previously found that p110 CUX1 acts as a transcription factor to upregulate DNA damage response proteins, they recently discovered that p200 CUX1 plays a direct role in BER, aiding the 8-OxoGuanine DNA Glycosylase, OGG1[92] (**Fig. 6**: right). This discovery could explain CUX1 as a haploinsufficient tumor suppressor. Insufficient CUX1 in cells aids in tumor initiation, as it would lead to an accumulation of mutations that could aid the tumor formation.

Transcriptional Role

CUX1 codes for many protein isoforms, the most common one being the full-length protein, often called p200 CUX1. Initially, p200 CUX1 was believed to act as transcriptional repressor that downregulates expression of genes expressed in terminally differentiated cells; however, this did not make sense as p200 CUX1 was found to be expressed in fully differentiated cells[80 88]. As p200 CUX1 exhibits extremely fast DNA binding kinetics, binding DNA rapidly but only transiently, it could act as a repressor by competing for occupancy of DNA binding sites overlapping with those for transcriptional activators[93].

Additionally, p200 CUX1 can be cleaved between CR1 and CR2 to yield p110 CUX1, which can both positively and negatively affect transcription. CUX1 p110 promotes tumor progression through its many roles in conferring advantages to cancer cells, including transcriptional roles stimulating cell cycle progression and cell proliferation, strengthening the spindle assembly checkpoint, and promoting cell migration and invasion and resistance to apoptosis[94 95]. One of its most important roles is in regulating transcription of DNA damage response genes, including ATM and ATR. When CUX1 is inhibited, there is decreased transcription of these genes as well as decreased signaling in the following DNA damage response pathway, leading to increased strand breaks. CUX1 p110 directly binds to promoters and increases the transcription of DNA damage response genes when bound. According to Vadnais et al, knockdown of CUX1 sensitizes cancer cells to irradiation as it prevents efficient ATM/ATR DNA damage response signaling[93]. In addition, CUX1 was found to promote

expression of cell cycle checkpoint genes, like ATM, ATR, CHK1 and CHK2, as well as DNA damage sensors NBS1, TopBP1, and RPA that activate ATM/ATR. In accordance with these findings, CUX1 knockdown was shown to sensitize cells to UV and ionizing radiations[96]. Thus CUX1 contributes to maintaining genome integrity by ensuring constant levels of DNA damage response proteins in cells prior to genotoxic stress.

Direct Role in DNA Repair (CR1CR2-NLS)

Recently researchers are investigating another use for these transcription factors as aids to glycosylase activity. Some believe that transcription factors could influence the binding of glycosylase to the lesion in DNA; in addition to CUX1 and OGG1, transcription factors YB-1 and HMGB1 stimulate glycosylases NEIL2 and APE1, respectively[97 98]. Specifically, Cut repeats stimulate OGG1, which initiates BER. Cut repeats are recruited to DNA damage site and also bind to PARP and KU in the BER and NHEJ pathways respectively[99]. These findings promote a new view into how these transcription factors can influence Base Excision Repair, and how they have potential to become therapeutic targets in cancer patients.

Dr. Nepveu's laboratory has found that p200 CUX1 has a direct role in DNA repair with regards to oxidative damage through its three Cut domains. CUX1 Cut domains bind OGG1 and stimulate its binding to DNA as well as its glycosylase and AP-lyase activities[92 100]. Each of the three Cut repeats was shown to stimulate OGG1. In agreement with these findings, Dr. Zubaidah Ramdzan found that when mouse embryo fibroblasts from Cux1 knockout mice were placed in 20% oxygen, they senesced immediately, even though they proliferated well in 3% oxygen[92]. This experiment strengthened the idea that CUX1 was indeed important in the repair of oxidative DNA damage. On the other hand, higher CUX1 expression in RAS-driven cancer cells, which produce higher levels of ROS, enabled rapid repair of oxidative DNA damage, preventing cellular senescence and allowing proliferation[92]. Suggested mechanisms of action are either that Cut repeats make the 8-oxoG sites more accessible to OGG1 or that they aid OGG1 in restructuring the DNA to make sites more accessible[100]. Cut repeats could also render the OGG1 active by changing its conformation upon binding[100]. Importantly, we have observed that Cut repeats also stimulate APE1 activity, in a cleavage assay performed by Simran Kaur[112]. This observation strengthens the claim that CUX1 holds an important role in BER (**Fig. 6**).

Measuring DNA Damage and Repair in Response to Irradiation

Evolution of the Comet Assay: A Brief Overview

In order to identify proteins involved in DNA repair and in DNA damage response pathways, DNA damage was measured through different methods. The most common method currently is single cell gel electrophoresis (SCGE), or the comet assay. Most methods depend on the idea that upon denaturation strand breaks generate short DNA strands that do not migrate with the bulk of nuclear DNA but migrate farther through a gel, and that shorter DNA strands are indicative of greater frequency of damage.

The comet assay is most interesting in measuring DNA damage, as it can be used to measure specific types of DNA lesions, whether it be present as single strand breaks (SSBs) or as double strand breaks (DSBs). With some adaptations, the comet assay can show DNA SSBs, DSBs, cross-linking, base damage, apoptotic nuclei, AP sites and oxidative DNA damage[101]. Most commonly, it is favored for its ability to detect DNA single and double-stranded breaks.

Originally, in 1984 Ostling and Johanson developed the initial form of the comet assay, as they sought to analyze the relaxation of supercoiled DNA caused by single-strand breaks[102]. In 1988, Singh further developed the comet assay using alkaline conditions[103]. Later, Olive developed a combination of electrophoresis and fluorescence microscopy to visualize DNA strand breaks[104]. This was based on the theory that shorter strands were caused by a greater number of breaks—thus a sign of greater DNA damage—and would migrate farther on the gel. Through assigning a score relating the amount of DNA damage to the amount of measured DNA and migration distance, the amount of damage in the cell could be assessed, and thus differences in DNA damage could be compared with regards to various treatments. The standard measurement of DNA damage visualized as comets is the comet tail moment.

As stated previously, the comet assay can be manipulated to measure different types of DNA damage. For example, maintaining cells at a neutral pH (7-8) allows detection of DNA double strand breaks, independent of single strand breaks[105]. Maintaining the cells at alkaline pH reveals more damage, since due to high pH alkali-labile sites will convert to single-strand breaks and DNA will be denatured, so that single-strand DNA flanked by two single-strand breaks will be released[105]. Thus, with alkaline comet assays at pH 10, scientists can observe single-stranded and double-stranded breaks alike. In addition, comet assays at pH >13 detect SSBs and DSBs, abasic sites, and many types of altered bases susceptible to high pH[105]. On the other hand, comet

assays at pH 10 only detect SSBs and DSBs. Prior to electrophoresis, one can treat cells with a DNA glycosylase such as FPG and OGG1to detect specific types of altered bases[106]. Maintaining a comet assay at alkaline pH 10 with the addition of a glycosylase yields greater damage due to the cleaving of oxidative DNA lesions[106].

A comet assay can consist of treating or not a specific number of cells with either irradiation or drugs, and embedding them in agarose on a pre-coated *Trevigen* plate. The plates are then lysed at a specific pH—depending on whether an alkaline or a neutral comet is carried out. For the neutral pH variation, the plates are kept at high temperature when submerged in neutral lysis buffer. Next, the plates are submerged and electrophoresed in either neutral or alkaline electrophoresis buffer. Later, the plates are stained with propidium iodide, SYBR green, or SYBR gold and viewed under a confocal microscope. Images are taken of individual cells that are then scored as to the proportion of migrated damaged DNA (tail) to the intact DNA (head) via CometScore software (TriTeck Corp)[107]. Other softwares are available that will automatically determine the head and tail of the comet, but in our lab's case, we manually tell the software the different parts of the comet to score.

Traditionally, in the comet assay cells were irradiated or treated with drug in plates, prior to incubation at 37°C in an incubator to allow for repair of DNA damage and measurement of DNA damage at specific times. Then cells were trypsinized, washed in PBS, and embedded into agarose, the entire process taking roughly 15 minutes. Soon it became evident that repair of radiation-induced DNA damage happens very quickly, and as such, we had to limit the time it takes between recovery time point and plating cells into agarose. We irradiated the cells only after trypsinizing and washing them in PBS. Thus we were better equipped to observe DNA repair in the first minutes after irradiation. This thesis will outline the intensive troubleshooting that was required to observe the rapid DNA repair time of cancer cells post-irradiation through the comet assay. The specific protocols for comet assays at each pH (pH 14, pH 10 +/- glycosylase, pH 8) will also be discussed.

Other Methods of Measuring DNA Damage Repair

There are additionally other, less common methods to measure DNA damage. Micronuclei detection assays measure DNA fragmentation[108]. Halo assays are used to detect changes in genomic structure when cells are treated with irradiation[109]. Damage is measured by calculation of the "halo area" which surrounds the nucleoid when visualized under the

microscope[109]. Similarly to the comet assay, to assess damage due to single strand breaks, the assay is done under alkaline conditions. TUNEL (Terminal deoxyribonucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling) assays involve tagging the ends of DNA broken strands with a fluorescent marker[110]. The amount of labeling observed is an indicator of the amount of DNA strand breaks that leaked from the apoptotic cells[110]. There had been some concerns that the TUNEL assay was detecting necrotic cells as well, but since then improvements have been made so that it only detects cells in the last phase of apoptosis. Other assays used that measure initial DDR signaling are the γ-H2AX and 53BP1 foci assays. Since H2AX is phosphorylated by ATM to form γ-H2AX foci at the onset of DSBs, an antibody against γ-H2AX can be used to measure DNA damage in a cell. Since 53BP1 is also recruited to the double-strand break, albeit later, one can also measure DNA damage by measuring amount of 53BP1 in a cell with a corresponding antibody. Additionally, there exists assays that explicitly measure the repair of a specific lesion. In fact, recently Nagel et al. detailed an assay that measures DNA repair capacity in many pathways[111]. By transfecting cells with plasmids containing a lesion, such as 8-oxoguanine or O^6-methylguanine, or mismatch or DSB, alongside a fluorophore, they can measure repair activity of BER, MGMT, MMR, or NHEJ, respectively, as the defects are repaired[111].

Rationale

The p200 CUX1 protein was shown to function as an accessory factor that stimulates OGG1[92 100]. Moreover, unpublished observations indicate that p200 CUX1, via its Cut repeats, can also stimulate APE1[112]. Radiotherapy consists in submitting tumors to ionizing radiation. As detailed earlier, ionizing radiation causes DSBs directly and increases the levels of ROS, which produce a number of oxidative DNA lesions including 8-oxoguanine and AP sites, the repair of which is initiated by OGG1 and APE1, respectively. We postulated that CUX1, and in particular the Cut repeats, could represent a therapeutic target: a drug that inhibits the stimulation of OGG1 and APE1 by Cut repeats may be able to sensitize cancer cells to radiotherapy. To validate CUX1 as a therapeutic target, we initiated a series of experiments to test the effect of CUX1 knockdown and overexpression on the resistance of cancer cells to ionizing radiation. The goal of my project was to choose and optimize the assays that would enable us to perform these experiments. In parallel, I performed preliminary experiments to investigate the effects of CUX1 knockdown and overexpression on the resistance of cancer cells to ionizing radiation and to other treatments that increase oxidative DNA damage.

Materials and Methods

Cell Culture

Human cell lines, (breast carcinomas Hs578T, MKN45, and MDA-MB-231; colorectal cancer HT29, DLD-1, and HCT116; glioblastoma U87, U251, and T98G; lung carcinoma HCC827 and A549; hTERT-immotalized RPE1) were cultured following provided instructions. Cell lines Hs578T, MKN45, MDA-MB-231, DLD-1, DKO-4 were maintained in Dulbecco's modified Eagle medium (DMEM)-high glucose, U251 and U87 in DMEM-F12, A549 and HCC827 in RPMI-1640 (Wisent), supplemented with 10% Fetal Bovine Serum (Tetracycline-free; Gibco), penicillin–streptomycin (Invitrogen), and maintained at 37°C, 5% CO_2 and atmospheric O_2.

Generation of Stable Cell Lines

Retroviruses were produced using 293VSV cells that were co-transfected with pLXSN-p200 CUX1-HA with packaging plasmids pVPack-GP and pVPack-VSV-G (Stratagene). Lentiviruses were produced by co-transfecting 293-FT cells with plasmids pLenti humanOGG1 (ThermoScientific), pLenti-CR1CR2-NLS-HA, pTRIPZ-DoxOn-shCUX1 plasmid (OpenBiosystems), packaging plasmid psPAX2 and envelop plasmid pMD2G[88]. The medium of the transfected cells containing the retrovirus or lentivirus was collected for 5 and 3 days respectively, starting 48 hours post-transfection. Viruses were applied to cells along with 6 μg/ml polybrene and cells were centrifuged at 1200*g* for 1 h. Infected cells were selected and maintained with specific antibiotics, blasticidin, G418 or puromycin. Expression of CUX1-shRNA was induced in the stably infected pTRIPZ-DoxOn-shCUX1 cells by supplementing the growth media with 1 μg/ml of doxycycline. Cells grown in the absence of doxycycline were used as a control. Over-expression of different genes or knockdown of CUX1 was confirmed by immunoblot analysis.

Protein Extracts

Nuclear extracts were prepared using a procedure adapted from Lee et al.[113] Briefly, cells were submitted to three freeze/thaw cycles in Buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT). Nuclei were then resuspended in Buffer C (20 mM Hepes, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 420 mM $NaCl_2$, 0.2 mM EDTA) and incubated at 4 °C for 30

min. After 15 min of centrifugation, the supernatant was collected. Buffers A and C were supplemented with protease inhibitor mix tablet (Roche Applied Science).

Immunoblotting

Proteins extracts were resuspended in Laemmli buffer, boiled for 5 min, resolved by SDS-PAGE, and electrophoretically transferred to a PVDF membrane. Membranes were blocked in TBS-T (10 mM Tris, pH 8, 150 mM NaCl, 0.1% Tween X-100) containing 5% milk and 2% bovine serum albumin. Membranes were then incubated with primary antibodies diluted in TBS-T, washed in TBS-T, and incubated with species-specific secondary antibodies conjugated to horseradish peroxidase for 45 min at room temperature. Proteins were then visualized using the ECL system of Amersham Biosciences according to the manufacturer's instructions. The following antibodies and dilutions were used: anti-CUX1 861 (1/1000), anti-OGG1 (Pierce, PA1-31402; 1:1000), and anti-tubulin (Sigma, T6557; 1:1000).

Radiation, TH588c, Clonogenic Survival Assay

Clonogenic efficiency of irradiated cells was measured as described previously[114]. Briefly, cells were exposed to ionizing irradiation at doses of 1, 2, 4, 5, and 8 Gy using an X-ray source biological irradiator (Rad-Source RS2000), and/or treated with the MTH1 inhibitor, TH588c. 200 cells were then plated in 6-well plates in triplicate. After 10-14 days of incubation, cells were washed with phosphate-buffered saline (PBS), fixed with cold methanol for 20 min then stained with 0.1% crystal violet (Acros Organics) in 20% methanol for 30 min. The number of colonies with 50 cells or more was counted. Clonogenic efficiency is the percentage of seeded cells that gave rise to clones under control conditions (empty vector cells with no irradiation). The reported values are the averages ± standard deviations.

Methyl-^{14}C Thymidine Incorporation

DLD-1, T98G, MDA-MB-231 cancer cells exposed to ionizing irradiation (0, 1, 2, or 4 Gy) were plated at a density of 4 x10^3 cells per well in 96-well Cytostar-T scintillating microplates (PerkinElmer). Cells were incubated in 100 µl of media with 0.5µCi/ml of methyl-^{14}C thymidine. The incorporated thymidine was quantified twice a day with a microplate counter (MicroBeta2, PerkinElmer). Each time point was done in triplicate, and the averages ± standard deviations were calculated. This assay was repeated with testing of OGG1 inhibitors, where DLD-1 cells were exposed to vehicle (DMSO) or 10 µM of chemical molecules (Chembridge

5245457 and 5552704) for 2 h prior to ionizing irradiation (2Gy), and plated in the presence of vehicle or 10 μM chemical molecules.

Single-Cell Gel Electrophoresis

To measure DNA strand breaks in T98G and DLD-1 cell lines overexpressing CR1CR2-NLS, OGG1, or repressing CUX1 expression, single cell electrophoresis (comet assays) was carried out using pre-coated slides (Trevigen, MD, USA). Total strand breaks were conducted in alkaline pH as described in Olive and Banath (2006)[115]. Single and double DNA strand breaks as well as oxidative DNA damage were conducted using formamidopyrimidine-DNA glycosylase (FPG) enzyme (or OGG1) in pH 10 as described by Collins, Duthie et al. (1993)[116]. Cell lines were irradiated (0, 10 GY, 40 GY), and given different incubation times post-irradiation (0, 15 min, 30 min, 60 min, 360 min), using ice to prevent further DNA repair. These cells were embedded in agarose onto Trevigen plates and lysed either at pH 8 or pH 10. These plates were then either placed in neutral or alkaline electrophoresis buffer and electrophoresis was run for 20 min. After electrophoresis, the plates were stained with propidium iodide (or SYBR GREEN or GOLD) and visualized with Axiovert 200M microscope with an LSM 510 laser module (Zweiss), 30 images taken per well. Comet tail moments were scored for at least 50 cells per condition, based on the proportion of the tail (DNA damage) to the head (undamaged DNA) via CometScore software (TriTeck Corp). The comet tail moment measures the length and amount of DNA strand breaks (tail) that disperse one-dimensionally through the gel, against the intact DNA that remains in place.

Statistical Analysis

Statistical analyses were calculated using the Student's t-test. Differences between groups were considered significant at * $p < 0.05$, ** $p < 0.01$, and *** $p<0.001$. Error bars indicate the SEM.

Results

Doxycycline Creates an Artifact in the Methyl-^{14}C Thymidine Incorporation Assay

In order to investigate the effect of CUX1 knockdown on proliferation of cancer cells, cancer cell lines were stably infected with a doxycycline-inducible shCUX1 pTripz vector. Then cell lines were treated with doxycycline for 72 hours, and all cells were irradiated and submitted to the Methyl-^{14}C thymidine incorporation assay. ^{3}H-labeled thymidine was not used as it labels RNA as well as DNA, while thymidine labeled on its methyl group will solely be incorporated in DNA. Our lab uses Methyl-^{14}C-labeled thymidine specifically so that we can solely measure DNA synthesis (Perkin Elmer).

Here 4,000 cells were plated per well in a 96-well scintillating counter plate and were left to proliferate for several days, with readings done at 24-hour time points. A surprising event occurred when the experiment was carried out in MKN-45 and Hs578T cell lines. The thymidine incorporation of the pTripz shCUX1 vector cell line with doxycycline increased at an alarming rate. In fact, by the end of day 3, the doxycycline-induced shCUX1 cell lines had increased their thymidine incorporation readings by nearly 5 times compared to their non-doxed counterparts (**Fig. 8**: Hs578T). Even after irradiation there was a noticeable difference between the two, although it was significantly decreased.

Surprised by this result, we considered that the doxycycline added to the wells might have been the root of this effect. We proceeded to test Hs578T and DLD-1 cell lines carrying an empty vector that would not be affected by addition of doxycycline. 4,000 cells were plated per well in a 96-well scintillating counter plate, with either 0, 1, 2.5, or 5 ug/ul of doxycycline. While there was no evident correlation between amount of doxycline added and amount of thymidine incorporation read, there was great increase in thymidine incorporation read with any amount of doxycycline added (**Fig. 9**: top—DLD-1, bottom—Hs578T).

Knowing that doxycycline created an undesirable effect, we sought to remove this effect by reducing the number of cells plated per well. In an Hs578T cell line carrying an empty vector, we attempted the assay with either 500, 1000, and 4000 cells/well (**Fig. 10**). Unfortunately we still see an increase in thymidine incorporation even when cells are plated at lower density, although for some reason I do not fully understand, the effect takes longer to manifest at lower concentrations, appearing after at least 48 hours in lower concentrations compared to within the first 24 hours.

In light of these findings, I concluded that the thymidine incorporation assay cannot be used when cells must be treated with doxycycline.

Amount of DNA Damage Depends on Cell Line (Comet Assay)

Single-cell gel electrophoresis (comet) assay was used to assay DNA strand break repair in cancer cells, specifically, damage due to ionizing radiation. As ionizing radiations produce a wide spectrum of DNA damage, different variations of the comet assay must be employed to measure various types of DNA lesions. Comet assay at pH 14 measures a wide range of damage, from alkylated bases to oxidative damage to double and single- stranded breaks. Comet assay at pH 10 measures single- and double- stranded breaks. Comet assay at pH 10 with prior treatment of cells with a DNA glycosylase like FPG or OGG1 will, in addition, reveal the presence of oxidized bases. pH 8, according to Collins, measures solely double-stranded DNA breaks[99].

In first setting up this assay, we sought to identify a cell line that could be best used for the comet assay, as well as the minimum dose of irradiation necessary to visualize DNA damage and corresponding DNA repair. DNA damage in Hs578T, DLD-1, and T98G parental cell lines was measured at varying doses of radiation using the comet assay. Surprisingly DLD-1 and Hs578T parental cell lines exhibited high DNA damage even when untreated with irradiation (**Fig. 11**: Compare A and C with B). We assumed that this could be due to them carrying an activated RAS oncogene, which causes the production of reactive oxygen species and consequently, oxidative DNA damage. While DLD-1 and Hs578T exhibited high damage in their non-treated samples, T98G cell line virtually showed no DNA damage (**Fig. 11**: B). In addition, it was observed that the longer a cell line was kept in culture, the more likely it was to exhibit high damage in the non-treated sample. As Hs578T gave inconsistent data, I chose to keep using DLD-1 and T98G cell lines in my future comet assay experiments.

DNA Damage Repair due to Ionization Radiation Occurs Rapidly

As we began experiments looking at irradiation-induced DNA damage recovery, we found a new problem: cells repair the DNA damage very rapidly. Following Olive's and Collins' protocols for the pH 14 and pH 10 +/- glycosylase comet assays[101], respectively, we were irradiating cells on plates, allowing them to recover at 37°C for a set amount of time, and then trypsinizing and PBS-washing them before embedding them in agarose, keeping samples on ice whenever possible to avoid further recovery. We realized that the process of trypsinizing and

washing of cells in PBS gave the samples approximately an extra 15 minutes recovery time. As a consequence, in my first experiments I failed to detect much DNA damage even after irradiation at high doses (data not shown). Therefore, I needed to optimize the protocol to be able to measure DNA damage as fast as possible following irradiation. In order to give the samples the least extra recovery time possible, especially the samples labeled "0 min recovery", we investigated different methods of irradiation, comparing comet tail moment (DNA damage) of samples that were either irradiated on plates, irradiated in tubes after trypsinization and resuspension in PBS, or irradiated after being trypsinized, washed and embedded in agarose (**Fig. 12**: B). We found that irradiating on agarose revealed greater DNA damage than on the plate (**Fig. 12 B**: Compare experiment #s 2 to 3, 5 to 6, 9 to 10, and 13 to 14). Additionally we found that IR of cells in PBS suspension and of cells in agarose yielded similar amount of DNA damage (**Fig. 12**: B: Compare experiment #s 10 to 11). Since IR of cells in agarose or in PBS suspension yielded greater DNA damage than IR of cells in plate, and since IR of cells in agarose is simpler than of those in PBS suspension, we chose to IR cells in agarose to produce the "No Recovery" samples in future comet assay experiments.

Interestingly, we pursued another comet assay, this time looking at cell recovery in PBS suspension (**Fig. 12**: A). Here we had both irradiated (10 Gy) and non-irradiated cell samples sit in PBS suspension for the same recovery times (0, 30, and 260 min). Unexpectedly, we found that right after trypsinization and resuspension in PBS, cells had high damage, even without IR. Within 30 minutes, significant damage was repaired in the untreated cell line. Whether this difference in damage is due to trypsin stimulating something that repairs damage, or due to the trypsinization-resuspension process causing initial stress and damage to the cells, or simply due to an artifact is unclear. What we do realize is that DLD-1 cell suspensions in PBS can be kept in a 37°C water bath up to four hours without increase in DNA damage. This experiment also made us realize the importance of trypsinizing samples at the same time, ensuring that no sample is remaining in PBS suspension longer than another. Samples should be irradiated at different times, depending on their specific time of recovery. For example, a 4-hour recovery sample would be irradiated 3 hours before a 1-hour recovery sample, so that both will be trypsinized at the same time.

In addition, to avoid rapid repair of irradiation-induced DNA damage, "0 min recovery" post-irradiation samples were irradiated after being embedded in agarose, and for short recovery timepoints, cells were irradiated in eppendorf tubes after being trypsinized and washed in PBS,

with recovery being done in the tubes in a 37°C water bath. As cells become less viable if kept in PBS suspension for long periods of time (data not shown), and since we also noticed that there was little difference between irradiating on plate and in tube for longer recovery time-points, we concluded that we would keep irradiating cells on plates for the longer recovery time points (1 and 4 hours).

In the case of the neutral comet assay, which detects only double-strand DNA breaks, we concluded that 10 Gy was simply not enough to induce sufficient double-stranded DNA breaks (data not shown). We settled on 60 Gy in order to allow for a sufficient number of recovery time points to observe the effect of CUX1 on DNA repair.

DNA-Binding Stains Affect Fluorescence Intensity of Comet Assay

Additionally, we explored three different DNA stains. Originally our lab used the Propidium Iodide stain on agarose slides, and used a confocal microscope to take images. Due to a technical and accessibility issue, we had to adapt to using a wide-field microscope to take images. When viewed under a wide-field microscope, the propidium iodide stained slides had high background, which was only exacerbated when the slides were dried (**Fig. 13**: Compare images A and C). According to Trevigen, two DNA stains for comet are preferred due to their high signal: background ratio: SYBR Gold and SYBR Green.

A comet assay was done using DLD-1 shCUX1 cells, treated or not with doxycycline, irradiated at 10 Gy and allowed to recover at 0 and 60 minutes. As a control, the same cells were not irradiated. The experiment samples were quadrupled to allow for two different stains and two different slide preparation methods: SYBR Green and Propidium iodide, and with or without ethanol-drying of slides. 30-50 images were taken to allow for 50-100 comets to be analyzed (**Fig. 13**). Surprisingly, both stains do not show exactly the same result, even though they are the same samples. We realized that this could be explained by the fact that propidium iodide has a greater affinity for single stranded DNA than SYBR green does, according to Trevigen. In addition, ethanol-dried slides—recommended when using the Axiovert wide-field microscope—stained with propidium iodide had very high background (**Fig. 13**: Compare C with A). This high background affected the accuracy of comet tail measurement, as can be seen when comparing comet tail moment at "10 Gy 0 h" recovery time (**Fig 13**: Compare B and D). This was not true for SYBR Green. In fact, when the slides were dried with ethanol prior to staining with SYBR Green, they yielded an even sharper image than when they were left undried (**Fig. 13**: Compare

G with E, and G with C). Because of this stark difference in image quality and comet tail moment measurement, and because ethanol-dried slides can be stored for long periods of time, we concluded that SYBR green staining would be preferable over PI.

Another DNA stain—also the most expensive—is SYBR Gold, which has a great signal: background ratio (>1000) as opposed to propidium iodide (~20), and has a single strand to double strand DNA fluorescence ratio (0.84) that is more reflective of the one propidium iodide has (0.93). This is compared to SYBR green which has a lower ss:dsDNA fluorescence ratio of 0.57. In fact, later I will discuss a comet assay performed with T98G pTripz shCUX1 (**Fig. 23**), in which using SYBR Gold staining showed more damage overall than when done with PI staining, aiming to the higher specificity of SYBR Gold binding to DNA. It could be possible that with PI staining, we are losing some information as part of the comet tail might blend into the background. However, this event should not affect the experiment conclusion overall. For instance, whether the slides were stained with PI or with SYBR Gold, the conclusion was the same, CUX1 knockdown delayed DNA repair post-irradiation.

Ectopic Expression of CUX1 p110 Increases Survival

One goal of my project was to validate previous published observations made in the lab that the transcriptionally active CUX1 p110 isoform could promote proliferation of cancer cells and upregulation of DNA Damage Response genes[93 106 117 118]. Hs578T cell line was transfected with either an empty pRev vector or a pRev vector expressing p110 CUX1, and cells stably carrying the vector were selected using Hygromycin. A clonogenic assay was performed, where cells were irradiated at 0, 2, 5 Gy, and plated at 100 individual cells per 100 mm plate. Cancer cells that overexpressed p110 exhibited slightly increased clonogenic efficiency post-irradiation, but the difference was not statistically significant (**Fig. 14**).

Methyl-^{14}C thymidine incorporation assays were also performed to measure the effect of p110 CUX1 on DNA synthesis following irradiation. DLD-1 (**Fig. 15**: A), MKN-45 (**Fig. 15**: B), and Hs578T (**Fig. 15**: C) cancer cell lines were used to perform this assay. CUX1 p110 overexpression has a small protective effect in DLD-1 cells after irradiation at higher doses. However, there is no effect on non-treated MKN-45 cell line, and minimal effect on increasing DNA synthesis of the MKN-45 cell line in response to irradiation. Interestingly, in Hs578T cells, we notice that CUX1 p110 overexpression increases DNA synthesis in cells even without treatment with irradiation.

Ectopic Expression of Full Length p200 CUX1 Increases Survival of Tumor Cell Lines

I next tested the effect of p200 CUX1 ectopic expression on the clonogenic efficiency of cells following irradiation. We established populations of T98G, U87, DLD-1, and MDA-MB-231 cancer cell lines stably carrying a retroviral vector expressing p200 CUX1 or nothing, and performed clonogenic assays after irradiating the cells at 0, 0.5, 1, 2, or 4 Gy (**Fig. 16**). Interestingly, the different cell lines were affected to a different degree by the overexpression of p200 CUX1. p200 CUX1 overexpression significantly increased clonogenic efficiency of DLD-1, T98G, and MDA-MB-231 untreated cell lines (**Fig. 16**: A, C, D). However, p200 CUX1 increases DLD-1 cell line survival significantly only at low doses of irradiation, 0.5 Gy and 1 Gy (**Fig. 16**: C). In addition, p200 CUX1 overexpression significantly minimally increased survival in MDA-MB-231 and T98G cells post-irradiation at 4 Gy (**Fig. 16**: A and D). Importantly, p200 CUX1 also increased survival in U87 cells after treatment with irradiation (**Fig. 16**: C).

In order to determine whether the overall resistance to radiotherapy of certain cancer cell lines was linked to endogenous CUX1 expression, my colleague Zubaidah Ramdzan carried out an immunoblot with diverse parental cancer cell lines using an antibody against CUX1 (**Fig. 17**). These results suggest that certain cell lines are not affected by p200 CUX1 exogenous expression, as they already express a sufficient amount of endogenous CUX1 protein. In contrast, U87 cells were found to have the least amount of CUX1 expression, perhaps explaining the difference in survival between the p200 expressing cell line and the control U87 cell line.

OGG1 siRNA Knockdown Sensitizes Cancer Cells to Irradiation

Because CUX1 was previously shown to stimulate OGG1[92 100] and has been shown to promote resistance to radiotherapy, and because we were disturbed by Dr. Susan Wallace's findings that overexpression of OGG1 decreased repair and survival of TK6 cells[121 122], we set out to establish that cancer cells require OGG1 to repair damage caused by irradiation. Here we verify the effect of OGG1 knockdown on sensitivity of cancer cells to irradiation. DLD-1 colorectal and U251 glioblastoma tumor cells were transfected with three different types of siRNA acting against OGG1, or with a scramble siRNA. These cell lines were irradiated and subjected to a clonogenic assay (**Fig. 18**: C and D) and DLD-1 cells were subjected to a Methyl-^{14}C Thymidine Incorporation assay (**Fig. 18**: B). Results show that decrease in OGG1 expression decreases both clonogenic survival and thymidine incorporation of DLD-1 cancer cells following

irradiation (**Fig. 18**: C and B). Importantly, OGG1 knockdown decreases clonogenic survival in U251 cells both before and following irradiation (**Fig. 18**: D). Interestingly, Western blots carried out to verify successful OGG1 knockdown in U251 and DLD-1 tumor cells show less endogenous OGG1 in U251 than in DLD-1 cells, and the U251 cell lines transfected with OGG1 siRNA have less OGG1 than the DLD-1 lines transfected with OGG1 siRNA (**Fig. 18**: A). This discrepancy in OGG1 expression could indicate that the small amount of OGG1 left in DLD-1 cells after transfection would be enough to repair endogenous DNA damage in the untreated cell lines, compared to the U251 cell lines which did not have sufficient OGG1 to repair DNA damage, even when untreated.

Doxycycline-Inducible CUX1 Knockdown Increases Sensitivity to Ionizing Radiation in Cancer Cell Lines

Next, as a way to investigate cancer cell's dependence on CUX1 for repairing DNA damage, I tested the effect of CUX1 knockdown on the clonogenic efficiency of cancer cells following irradiation. Here breast cancer cell lines (Hs578T and MDA-MB-231), colorectal cancer cell lines (HT29, DLD-1, and HCT116), and glioblastoma cell lines (T98G and U251) were infected with lentiviruses created by co-transfecting 293-FT cells with pTRIPZ-DoxOn-shCUX1 plasmids. After the stable cancer cell lines were selected for with antibiotic puromycin, expression of CUX1-shRNA was induced with 1 μg/ml doxycycline for 72 hours. Cell lines were irradiated at 0, 1, 2, and 4Gy and submitted to a clonogenic assay. For most tumor cell lines, CUX1 knockdown reduces the clonogenic efficiency even in the absence of irradiation (**Fig. 19**). The one cell line that is not affected by CUX1 knockdown prior to irradiation is U251, which maintains its wild-type Ras protein. These results are in agreement with those of previous studies showing the synthetic lethality of CUX1 knockdown in cancer cells that harbor a KRAS or HRAS oncogene[11 92]. Indeed, many of the tested cancer cell lines carry an activating mutation in one of the RAS genes: Hs578T, MDA-MB-231, HT29, DLD-1, and HCT116. However, the T98G glioblastoma cells do not contain a mutant RAS gene, and yet are still affected by reduced expression of CUX1. In the absence of CUX1, oxidative DNA damage is not repaired as efficiently and ultimately, leads to cellular senescence[92]. It is possible that T98G cells also produce high ROS levels, despite not carrying a RAS oncogene. This remains to be verified.

Importantly, in all cancer cell lines mentioned, CUX1 knockdown further reduced the clonogenic efficiency of cancer cells after irradiation (**Fig. 19**). The combination of CUX1 knockdown and radiotherapy reduces the survival of cancer cells.

Ectopic Expression of Recombinant CUX1 CR1CR2-NLS Protein and OGG1 Increases Resistance to IR in Cancer Cell Lines

To verify that the role of CUX1 in conferring resistance to radiotherapy in cancer cells was indeed via its DNA repair function, and not solely on its transcriptional activity, we employed a lentiviral vector expressing a recombinant CUX1 protein containing the Cut repeats 1 and 2 fused to a nuclear localization signal, CR1CR2-NLS protein. This protein was previously shown in two cell types to be devoid of transcription activation potential and yet able to stimulate OGG1 *in vitro* and in cells[92 100]. We generated cell lines stably carrying a lentiviral vector expressing the CR1CR2-NLS or nothing (vector). These cells were then submitted to radiation and their resistance or sensitivity assessed using both a clonogenic assay and a Methyl-^{14}C Thymidine Incorporation assay. In clonogenic assays, overexpression of CR1CR2-NLS in MDA-MB-231 and T98G cancer cell lines significantly increased survival of MDA-MB231 and T98G cancer cells following a low dose of ionizing radiation (1 Gy) (**Fig. 20**: B and C). In Hs578T cells, only a modest protective effect was observed at 2 Gy (**Fig. 20**: A).

In the thymidine incorporation assays, CR1CR2-NLS appears to increase the capacity of cells to synthesize DNA following irradiation (**Fig. 21**: A, B, C, and D, 2 and 4 Gy, or 6 Gy). This is especially clear in the case of T98G (**Fig. 21**: A). No difference in thymidine incorporation was observed in non-treated T98G cells and in cells treated with 1 Gy (**Fig. 21**: A, first two panels). At 2 and 4 Gy, however, T98G cells carrying the empty vector exhibited a decrease in thymidine incorporation at 1 and 2 days, while cells ectopically expressing CR1CR2-NLS continued to synthesize DNA almost to the same levels as in non-treated cells (**Fig. 21**: A, last two panels). The results are not as clear in the case of MCF7 and Hs578T cells, because thymidine incorporation reaches a plateau after 1 day in the non-treated cells carrying the empty vector, but continues to go up in cells expressing CR1CR2-NLS (**Fig. 21**: B and C, first panel on the left). It is not clear why a plateau is reached so quickly with MCF7 and Hs578T cells, but this makes it difficult to reach a firm conclusion about the protective effect of CR1CR2-NLS in these cells. Similarly, overexpression of CR1CR2-NLS confers some resistance to DLD-1 cells post-irradiation, offering a minimal protection effect even at 6 Gy (**Fig. 21**: D).

Based on previous studies from our laboratory showing that CUX1, and in particular the Cut repeats within CUX1, stimulate OGG1, we hypothesized that the radio-protective effect of p200 CUX1 and CR1CR2-NLS might be due, at least in part, to their ability to stimulate OGG1[92 100]. To test this hypothesis, we engineered DLD-1 colorectal cancer cells and RPE1 "normal" retinal pigment epithelial cells stably carrying a lentiviral vector expressing either CR1CR2-NLS, OGG1 or nothing (vector). Following ionizing radiation, the clonogenic efficiency of each population was measured. Both CR1CR2-NLS and OGG1 increased the resistance of DLD-1 cells to 1 and 2 Gy radiation but not of RPE1 (**Fig. 22**: Compare A, 1 and 2 Gy, to B). A difference was also observed at 4 Gy in DLD-1 cells, but a statistical significance could not be ascertained because of the small number of colonies at this dose (**Fig. 22**: A, 4 Gy). Interestingly, an increase in clonogenic efficiency was also seen for non-treated DLD-1 cells (**Fig. 22**: A, 0 Gy). This is likely due to the fact that DLD-1 colorectal cancer cells carry a KRAS oncogene and endogenously produce high levels of reactive oxygen species that can cause oxidative DNA damage. In contrast, ectopic expression of OGG1 and CR1CR2-NLS had no effect on survival of RPE1, prior to or after irradiation (**Fig. 22**: B). I surmise that increased expression of CR1CR2-NLS or OGG1 help DLD-1 cells repair oxidative DNA damage and continue to proliferate in the presence of ROS. In summary, results from this experiment support the notion that p200 CUX1 and CR1CR2-NLS increase radio-resistance in cancer cells through their role as accessory factor that stimulate the enzymatic activities of OGG1.

CUX1 Knockdown Slows DNA repair in both T98G and DLD-1 Cancer Cell Lines

Since we have established now that CUX1 promotes survival and proliferation of cancer cells following irradiation, since CUX1 stimulates OGG1 which, when its expression is reduced, sensitizes cancer cells to irradiation as well, the next step to take would be to confirm its involvement in DNA repair, by measuring its effect on speed of DNA repair post-irradiation. It would stand then, that a cell line with less CUX1 would take longer to repair DNA damaged by irradiation. Thus we sought to investigate DNA damage recovery through a single-cell gel electrophoresis (comet) assay of T98G and DLD-1 cell lines, carrying a doxycycline-inducible CUX1 knockdown (**Figs. 23 and 24**). Experiments done with both cell lines show that overall cells with CUX1 knockdown have greater damage throughout the experiment. As had been previously shown, CUX1 knockdown led to a greater amount of DNA damage even without

treatment[100], consistent with the idea that certain cancer cells might depend on CUX1 to deal with constant oxidative DNA damage.

Briefly, in T98G cells, CUX1 knockdown only seemed to affect repair of double-strand breaks 1 hr and 4 hr post-irradiation at 60 Gy (**Fig. 23**: D). Additionally, CUX1 knockdown significantly delayed repair of total DNA damage (abasic sites, SSB, DSB, alkali-labile sites) in both DLD-1 and T98G cells at 15 min, 30 min, 4 hr post-IR at 10 Gy, when electrophoresed at pH > 13 (**Figs. 23 and 24**: A). CUX1 knockdown significantly delayed repair at 30 mins and 1 hr post-IR at 10 Gy, when electrophoresed at pH 10 (solely measures SSB and DSB) (**Fig. 23**: B). Additionally, when the FPG enzyme was added to the slides, greater DNA damage was observed throughout the experiment (**Figs. 23 and 24**: Compare C to B). With addition of FPG, CUX1 knockdown significantly delayed repair in both DLD-1 and T98G cell lines at 30 min and 1 hr. Overall these experiments done in two cell lines show that decreasing CUX1 expression does have a negative effect on speed of DNA repair.

MTH1 Inhibitor Further Decreases Survival in Cancer Cells When Used on Cell Lines that Express CUX1 Knockdown

The next experiment was inspired by Thomas Helleday's research on MTH1 in cancer cells[32 33]. MTH1 is involved in preventing misincorporation of oxidized nucleoside triphosphastes into DNA, hydrolyzing oxidized bases such as 8-oxo-dGTP, 8-oxo-dATP, 2-hydroxy-dATP, and 2-hydroxy rATP, to monophosphates (**Fig. 6**: MTH1 is hydrolyzing 8-oxo-dGTP to produce 8-oxo-dGMP, preventing its incorporation into DNA. If incorporated, into DNA, this would produce an 8-oxoguanine lesion, repaired either by MMR (MYH), or by BER (OGG1).) When MTH1 is inhibited, cancer cells accumulate reactive oxidative species and DNA damage, ultimately leading to their death. Helleday's lab discovered a few compounds that inhibited MTH1, notably TH287 and TH588[32]. We received this latter compound from their lab, and decided to test its effect on cancer cells that either overexpressed CUX1 or expressed a CUX1 knockdown. Without CUX1, cancer cells could rely on another pathway to repair oxidative damage caused by accelerated metabolism and irradiation, such as the MTH1 pathway. If inhibiting MTH1 and causing CUX1 knockdown further decreases viability of the cancer cells, then this would indicate synthetic lethality of both proteins' role in repairing oxidative DNA damage, further highlighting the importance of CUX1 in conferring radio-resistance through increased repair of oxidative DNA damage.

Due to their high metabolism, some cancer cells create high amounts of reactive oxidative species that then damage the DNA. They have coped with this high oxidative DNA damage by increasing their capacity to repair this damage through increasing activity of certain repair pathways. Among these are the MTH1 pathway as well as the Base Excision Repair pathway. If CUX1 knockdown were to further sensitize cancer cells to MTH1 inhibition, compared to effect of MTH1 inhibition on cancer cell lines with normal CUX1 expression, then this would show that the cancer dependence on CUX1 is linked to the necessity of repair oxidative DNA damage. The synthetic lethality of CUX1 and MTH1 would determine the importance of repair of oxo-8-guanine lesions, and suggest that CUX1 has an additional role in removal of these lesions. This ties into the idea of redundant repair pathways in cells.

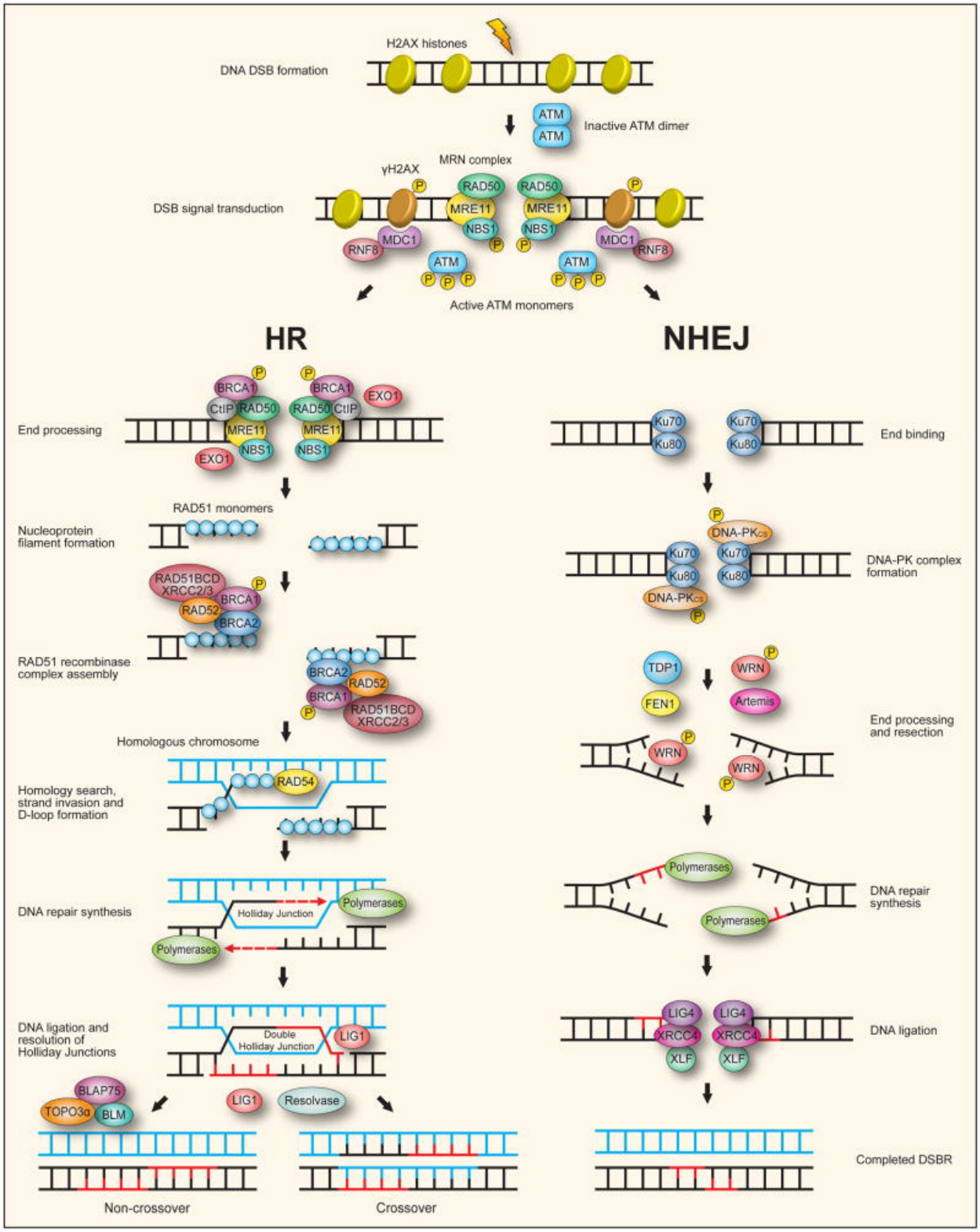
Here we treated DLD-1, Hs578T, and MDA-MB-231 (**Fig. 25**: A-C, respectively) cell lines carrying the pTripz vector containing doxycycline-inducible shCUX1 with or without TH588c (2, 4, or 5 μM). The cells were then plated in 6-well plates, and left to form colonies for 10-14 days. Colonies were stained with crystal violet stain solution, and then counted. Results indicate that while MTH1 inhibition barely has an effect at 2 μM on the control cancer cell line, MTH1 inhibition on a cancer cell line with decreased CUX1 expression has a negative impact on the cancer cell line survival, and an especially drastic negative effect in the MDA-MB-231 cell line (**Fig. 25**: C). Thus knockdown of CUX1 sensitizes cancer cells to MTH1, although the degree to which it does is dependent on the cell line.

Additionally, we observed that p200 CUX1 overexpression in cancer cell lines DLD-1, HCT116, Hs578T, and T98G (**Fig. 25**: D-G, respectively) increases survival of cells in response to MTH1 inhibition. Notice how p200 CUX1 overexpression is particularly able to rescue the oxidative stress caused by 2μM TH588c in HCT116 cells (**Fig. 25**: D). Similarly, we find that CR1CR2-NLS overexpression also increases survival in cell lines DLD-1 and HCT116 in response to MTH1 inhibition (**Fig. 25**: H and I). Interestingly, CR1CR2-NLS overexpression did **not** have an effect on cell survival in the DKO-4 cell line (**Fig. 25**: J). DKO-4 and DLD-1 cell lines make up a system of paired wild-type KRAS and KRAS mutant isogenic colon cancer cell lines, respectively. It is thus very interesting to note that without the added oxidative stress that a mutant RAS causes, overexpressing CUX1 has no effect on protecting DKO-4 from toxicity of MTH1 inhibitor TH588c, while it did promote resistance to TH588c in DLD-1 cells (**Fig. 25**: Compare H with J).

Figures

Figure 1: Double-Stranded DNA Repair via Homologous Recombination and Non-Homologous End-Joining

DNA double strand break repair pathways. A: Homologous recombination (HR) repair. MRE11-RAD50-NBS1 (MRN) complex recognizes double strand breaks (DSBs). This complex activates ATM kinase, which in turn initiates the full DNA damage response. CtIP-mediated nuclease activity is required for the end resection from 5' to 3', which leads to the formation of single-strand DNA (ssDNA). The exposed ssDNA is coated with DNA replication protein A (RPA) and activates the Ataxia Telangiectasia and Rad3-related protein (ATR) response to facilitate HR repair. Then RAD51 nucleoprotein filament is assembled, which replaces RPA-coated ssDNA, performs homology sequence searching, and mediates strand invasion. DSBs are restored by branch migration of this joint DNA molecule, DNA synthesis, ligation, and resolution of Holliday junctions; B: In NHEJ, the Ku70-80 heterodimer binds to the two DNA DSB ends, later recruiting DNA-PKcs to form a complex that brings the two ends together. Upon autophosphorylation, DNA-PKcs then recruit end processing enzymes like TDP1 to process the DSB ends into the ligatable 5'-P and 3'-OH termini. Polymerases later fill in the missing nucleotides, while NHEJ-specific nucleases like Artemis excise single-stranded overhangs. Similar enzymes might also include lesion-specific BER enzymes like tyrosyl-DNA phosphodiesterase 1 (TDP1), polynucleotide kinase (PNKP), apurinic/apyrimidinic endonuclease 1 (APE1), as well as exonuclease 1 (Exo1) and Werner Syndrome RecQ-Like Helicase (WRN). Importantly, recent studies suggest that Ku has an additional role of AP/deoxyribose 5'-phosphate (5'-dRP)-lyase activity, which would allow it to process abasic sites located close to the DSB in question that would otherwise prevent ligation[18 19]. Finally, the DNA Ligase 4—X-Ray Repair Complementing Defective Repair in Chinese Hamster Cells 4—XRCC4-like factor (Lig4-XRCC4-XLF) complex ligates the DNA ends. Importantly, in the absence of the Lig4-XRCC4 complex, the DNA can undergo alternative-NHEJ, which promotes chromosomal translocation.

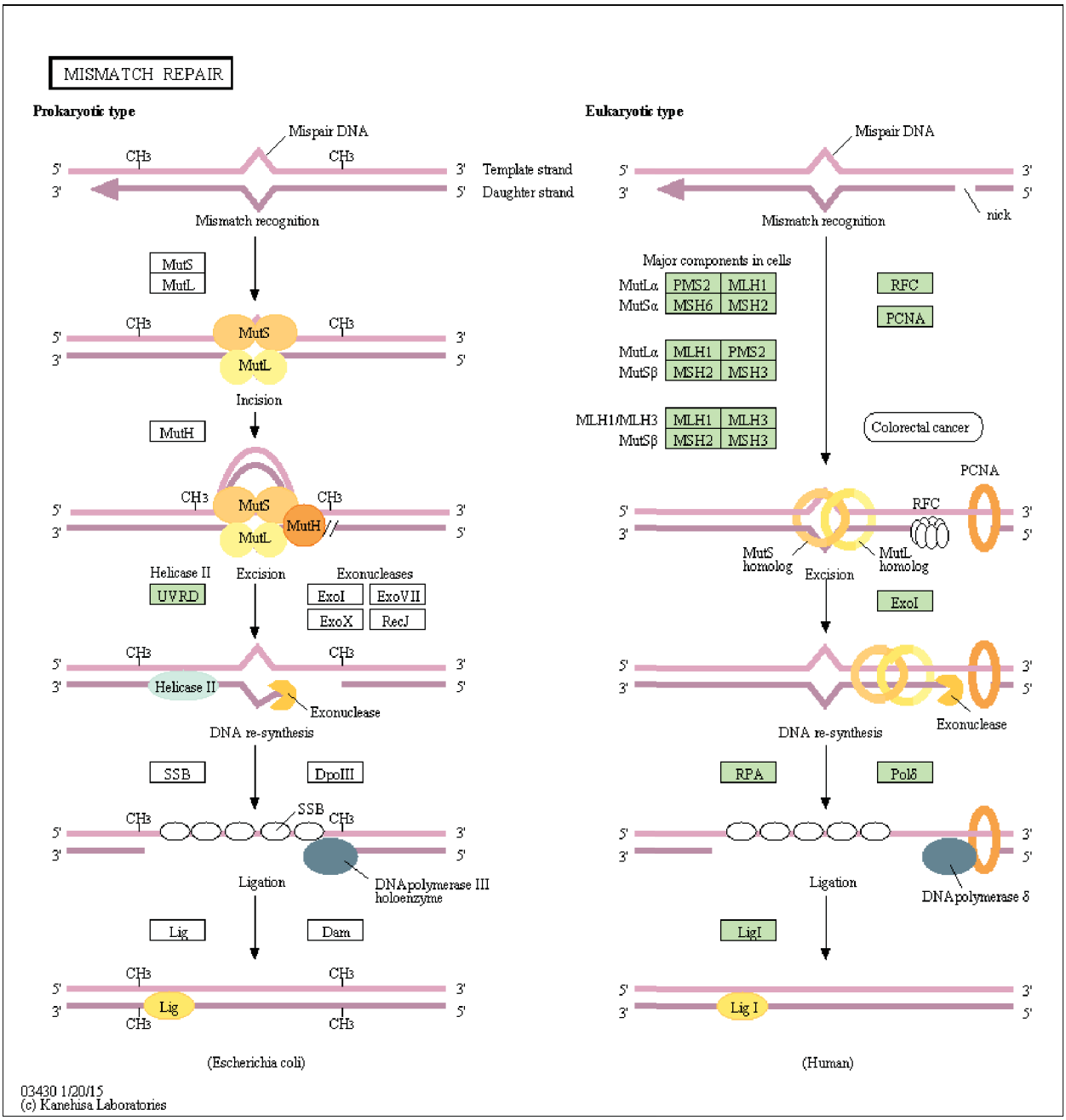


Jeppesen et al., 2011. Prog. Neurobiol. 94(2): 166-200.

Fig. 1

Figure 2: Mismatch Repair Pathway

DNA mismatch repair (MMR) is a highly conserved biological pathway that plays a key role in maintaining genomic stability. MMR corrects DNA mismatches generated during DNA replication, thereby preventing mutations from becoming permanent in dividing cells. MMR also suppresses homologous recombination between divergent DNA molecules and was recently shown to play a role in DNA damage signaling. Defects in MMR are associated with genome-wide instability, predisposition to certain types of cancer including Hereditary Nonpolyposis Colorectal Cancer (HNPCC), resistance to certain chemotherapeutic agents, and abnormalities in meiosis and sterility in mammalian systems. The *Escherichia coli* MMR pathway has been extensively studied and is well characterized. In *E. coli*, the mismatch-activated MutS-MutL-ATP complex licenses MutH to incise the nearest unmethylated GATC sequence. UvrD and an exonuclease generate a gap. This gap is filled by DNA pol III and DNA ligase. The GATC sites are then methylated by Dam. Several human MMR proteins have been identified based on their homology to *E. coli* MMR proteins. These include human homologs of MutS and MutL proteins. Although *E. coli* MutS and MutL proteins are homodimers, human MutS and MutL homologs are heterodimers. The role of hemimethylated dGATC sites as a signal for strand discrimination is not conserved from *E. coli* to human. Human MMR is thought to discriminate daughter and template strands by determining the orientation of PCNA loading on the DNA[21].

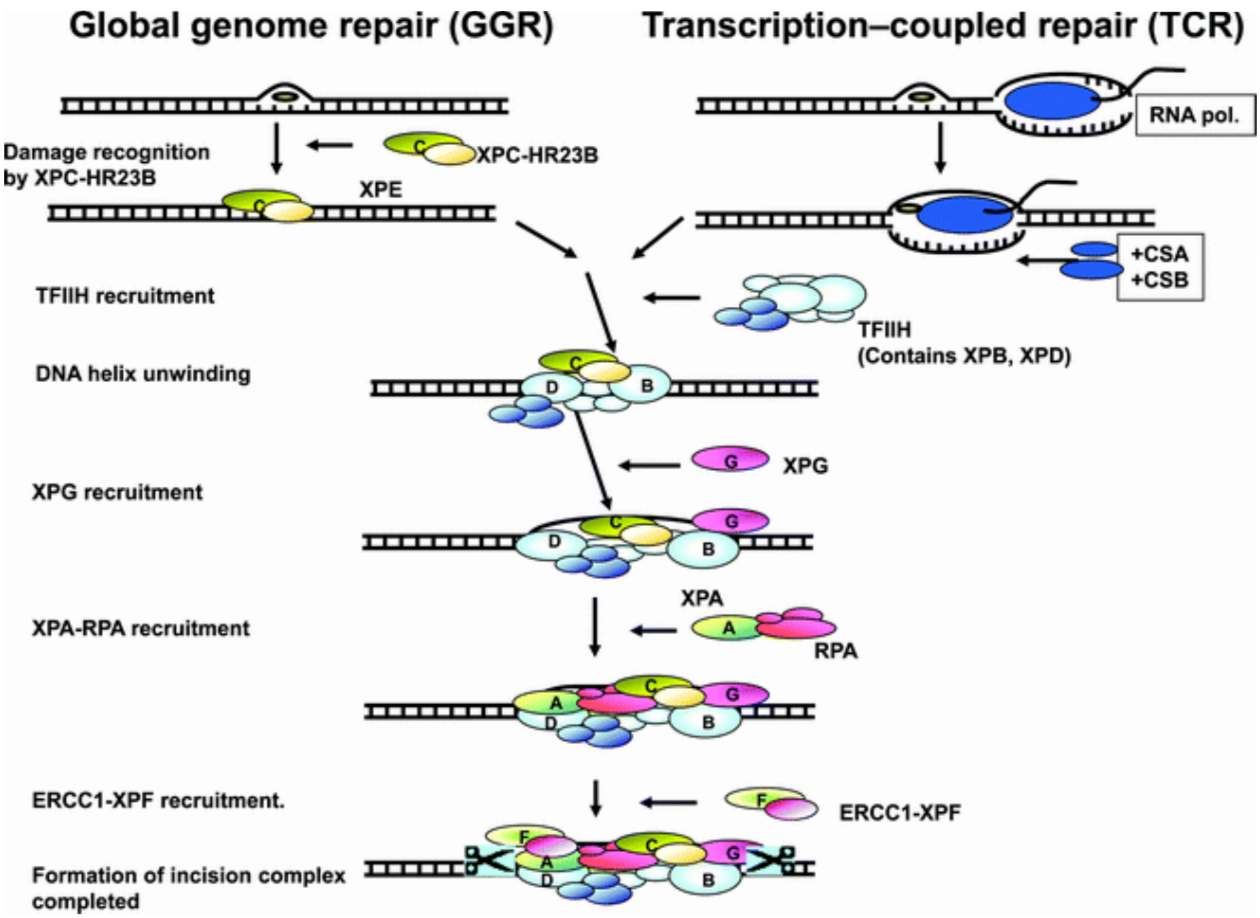


Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., and Tanabe, M.; KEGG as a reference resource for gene and protein annotation. 2016. Nucleic Acids Res. 44, D457-D462
Kanehisa, M. and Goto, S.; KEGG: Kyoto Encyclopedia of Genes and Genomes. 2000. Nucleic Acids Res. 28, 27-30

Fig. 2

Figure 3: Global-Genome and Transcription-Coupled Nucleotide Excision Repair

In the global genome nucleotide excision repair (GG-NER; left) sub-pathway, the damage sensor XPC constantly probes the DNA for helix-distorting lesions (step 1, left), which are recognized with the help of the UV–DDB (ultraviolet (UV) radiation–DNA damage-binding protein) complex, containing UV excision repair protein RAD23 homolog B (HR23B) (step 1, left). Upon binding of the XPC complex to the damage, RAD23B dissociates from the complex (step 2, left). In the transcription-coupled NER (TC-NER; right) sub-pathway, damage is indirectly recognized during transcript elongation by the stalling of RNA polymerase II (RNA Pol II) at a lesion (step 1, right). During transcript elongation Cockayne syndrome protein CSB transiently interacts with RNA Pol II and other proteins (step 2, right). Upon stalling at a lesion, the affinity of CSB for RNA Pol II increases and the Cockayne syndrome WD repeat protein CSA–CSB complex is formed, which probably results in reverse translocation (backtracking) of RNA Pol II that renders the DNA lesion accessible for repair. RNA Pol II and the nascent mRNA transcript are not depicted further. After damage recognition, the TFIIH (transcription initiation factor IIH) complex is recruited to the lesion in both GG-NER and TC-NER (step 3). In NER the XPG structure-specific endonuclease, either associated with TFIIH or separately, binds to the pre-incision NER complex (step 4). The helicase activity of TFIIH further opens the double helix around the lesion, and 5′–3′ unwinding of the DNA by the TFIIH basal transcription factor complex helicase subunit XPD verifies the existence of lesions with the help of the ATPase activity of the TFIIH XPB subunit and XPA, which binds to single-stranded, chemically altered nucleotides (step 5). In this step the single-stranded DNA binding protein replication protein A (RPA) is also recruited and coats the undamaged strand (step 5). XPA recruits a structure-specific endonuclease — the XPF–ERCC1 heterodimer, which is directed to the damaged strand by RPA to create an incision 5′ to the lesion (step 6). Once this 'point of no return' is reached, XPG is activated and cuts the damaged strand 3′ to the lesion, which excises the lesion within a 22–30 nucleotide-long strand (step 6). The trimeric proliferating cell nuclear antigen (PCNA) ring, which is directly loaded after the 5′ incision by XPF–ERCC1, recruits DNA Pol δ, DNA Pol κ or DNA Pol ε for gap-filling DNA synthesis. Gap filling can begin immediately after the 5′ incision is made. The NER reaction is completed through sealing the final nick by DNA ligase 1 or DNA ligase 3.



*H. Fassihi, Spotlight on 'xeroderma pigmentosum', Photo- chem. Photobiol. Sci., 2013, DOI: 10.*1039/C2PP25267H.

Fig. 3

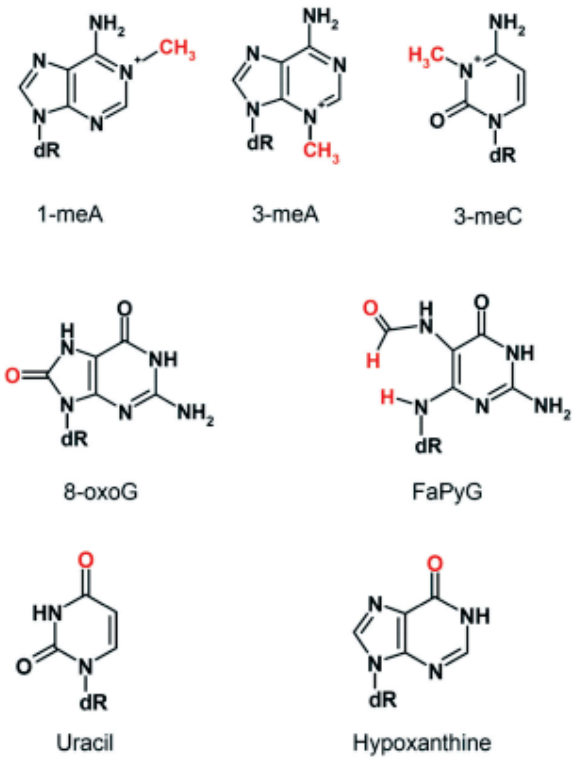
Figure 4: Mutated Bases Repaired by Base Excision Repair

Several damaged DNA bases are processed by the BER pathway. This figure shows the structures of many damaged bases that can be repaired by BER. 1-meA, 1-methyl adenine; 3-meA, 3- methyl adenine; 3-meC, 3-methyl cytosine; 8-oxoG, 8-oxoguanine; FaPyG, 2,6-diamino-4-hydroxy-5-formamidopyrimidine.

Top row: Major deleterious lesions formed by simple methylating agents in double-stranded (3-meA) and single-stranded DNA (1-meA and 3-meC). 3-meA is repaired solely by the BER pathway; and the lesions 1-meA and 3-meC can be demethylated by DNA dioxygenases.

Middle row: The two most abundant and best studied purine lesions, 8oxoG and FapyG, generated by oxidative stress and both targets of OGG1.

Bottom row: Uracil and hypoxanthine are examples of deaminated bases.

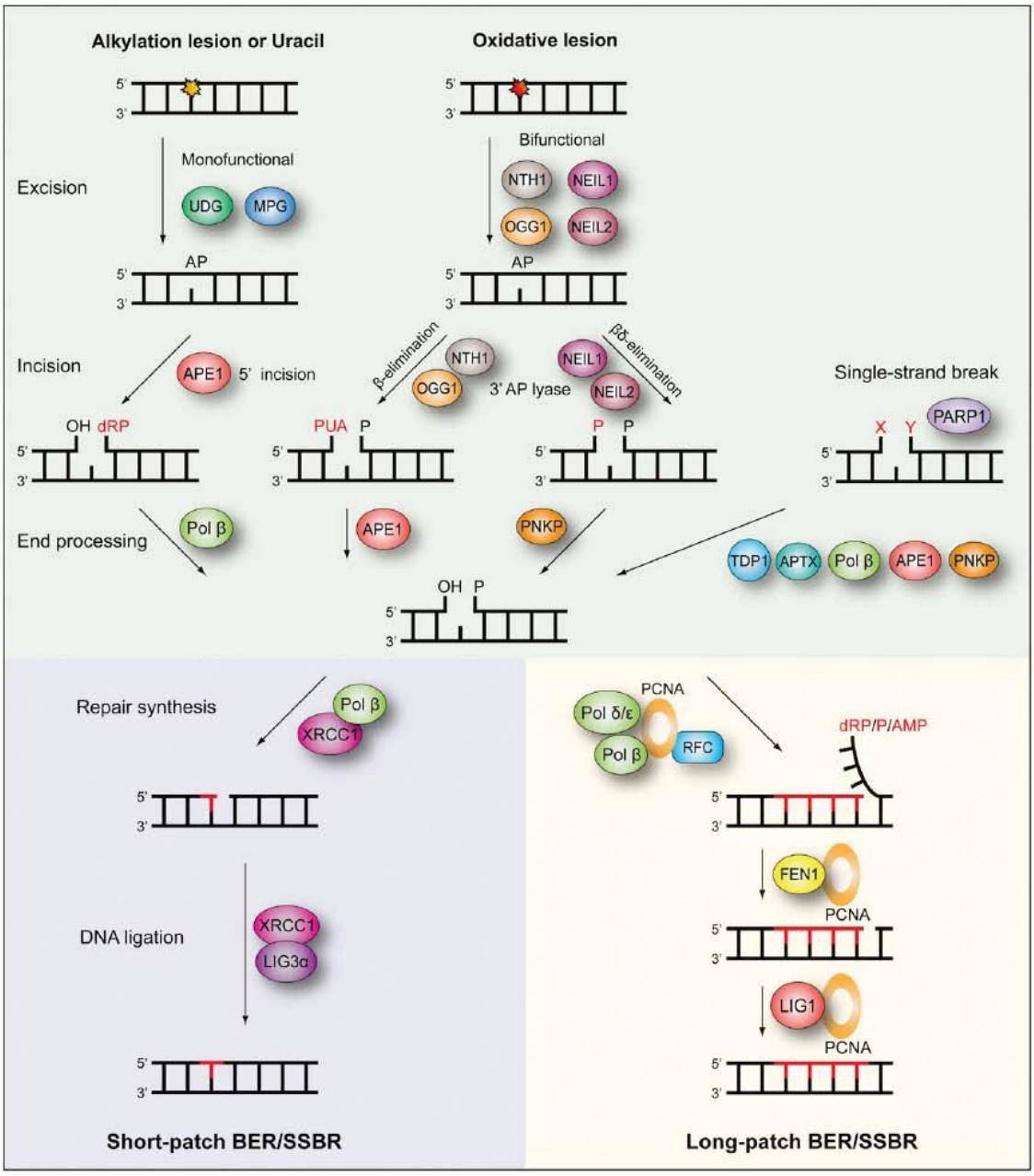


Robertson, AB, A Klungland, T Rognes, and I Leiros. "Dna Repair in Mammalian Cells: Base Excision Repair: the Long and Short of It." Cellular and Molecular Life Sciences: Cmls. 66.6 (2009): 981-93. Print.

Fig. 4

Figure 5: Short-patch and Long-patch Single-Stranded Break Base Excision Repair

Base excision repair (BER) is initiated by removal of the modified base by either a monofunctional or bifunctional DNA glycosylase to leave an apurinic/apyrimidinic (AP) site, often referred to as an abasic site. If excision is by either one of the monofunctional DNA glycosylases UDG or MPG, the following incision of the DNA backbone 5' to the AP site is performed by APE1. Excision by one of the bifunctional DNA glycosylases NTH1, OGG1, NEIL1 or NEIL2 is followed by incision 3' to the AP site via β - or β δ -elimination facilitated by the intrinsic 3' AP lyase activity of these enzymes. The resulting single-strand break will contain either a 3' or 5' obstructive terminus. End-processing is then performed by Pol β, APE1 or PNKP depending on the specific nature of the terminus. Single-strand breaks can also occur by other means and can contain simultaneous 3' and 5' obstructive termini. PARP1 recognizes these breaks and the end-processing may utilize the additional factors TDP1 and APTX. When end-processing has produced the necessary 3'-OH and 5'-P termini, DNA synthesis takes place via two sub-pathways, short-patch and long-patch. In short-patch BER/SSBR repair, synthesis of the single nucleotide gap is by Pol β aided by the XRCC1 scaffold, and subsequent ligation by LIG3a finishes the repair. In long-patch BER/SSBR, repair synthesis of the 2–13 nucleotide gap is by Pol β, and/or Pol δ/ε aided by PCNA and RFC. A resulting 5' flap is removed by FEN1 and the final ligation step is by LIG1.



Jeppesen et al., 2011. Prog. Neurobiol. 94(2): 166-200

Fig. 5

Figure 6: CUX1 Involvement in Repair of DNA damage by Reactive Oxidative Species

One of the most abundant lesions caused by reactive oxidative species (ROS) is 8-oxoguanine (8-oxoG). This figure shows three distinct mechanisms our cells have adopted to repair or prevent damage done by ROS. First, in the event of oxidized nucleosides, MTH1 can hydrolyze the oxidized triphosphates to monophosphates, preventing the altered base's incorporation into DNA (left side). If 8-oxoG is present in DNA, upon replication it incorrectly base pairs with adenine. MUTYH (aka MYH) will excise the mismatched adenine opposite of the 8-oxoG lesion (middle), and replace it with a C. The 8-oxo-guanine DNA glycosylase, OGG1, recognizes and excises the 8-oxo-guanine base in DNA (right side). The p200 CUX1 isoform was shown to stimulate the binding of OGG1 to 8-oxoguanine, and stimulate both its glycosylase and AP lyase activities[100] (right side).

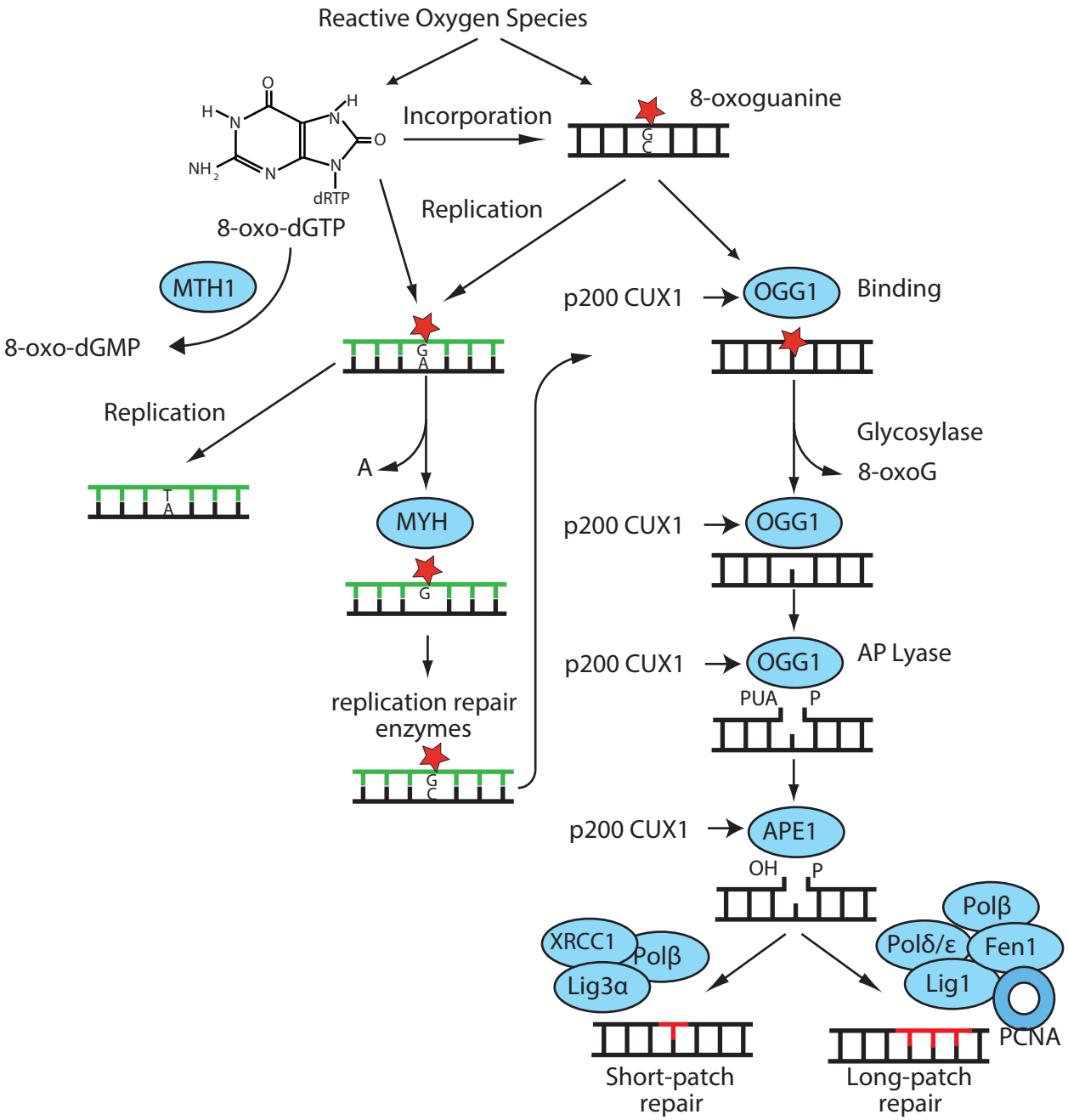

Reactive Oxygen Species
Incorporation
8-oxoguanine
dRTP
8-oxo-dGTP
Replication
MTH1
8-oxo-dGMP
p200 CUX1
OGG1
Binding
Replication
Glycosylase
8-oxoG
A
MYH
p200 CUX1
OGG1
p200 CUX1
OGG1
AP Lyase
PUA
P
replication repair
enzymes
p200 CUX1
APE1
OH
P
XRCC1
Polβ
Lig3α
Polβ
Polδ/ε
Fen1
Lig1
PCNA
Short-patch
repair
Long-patch
repair

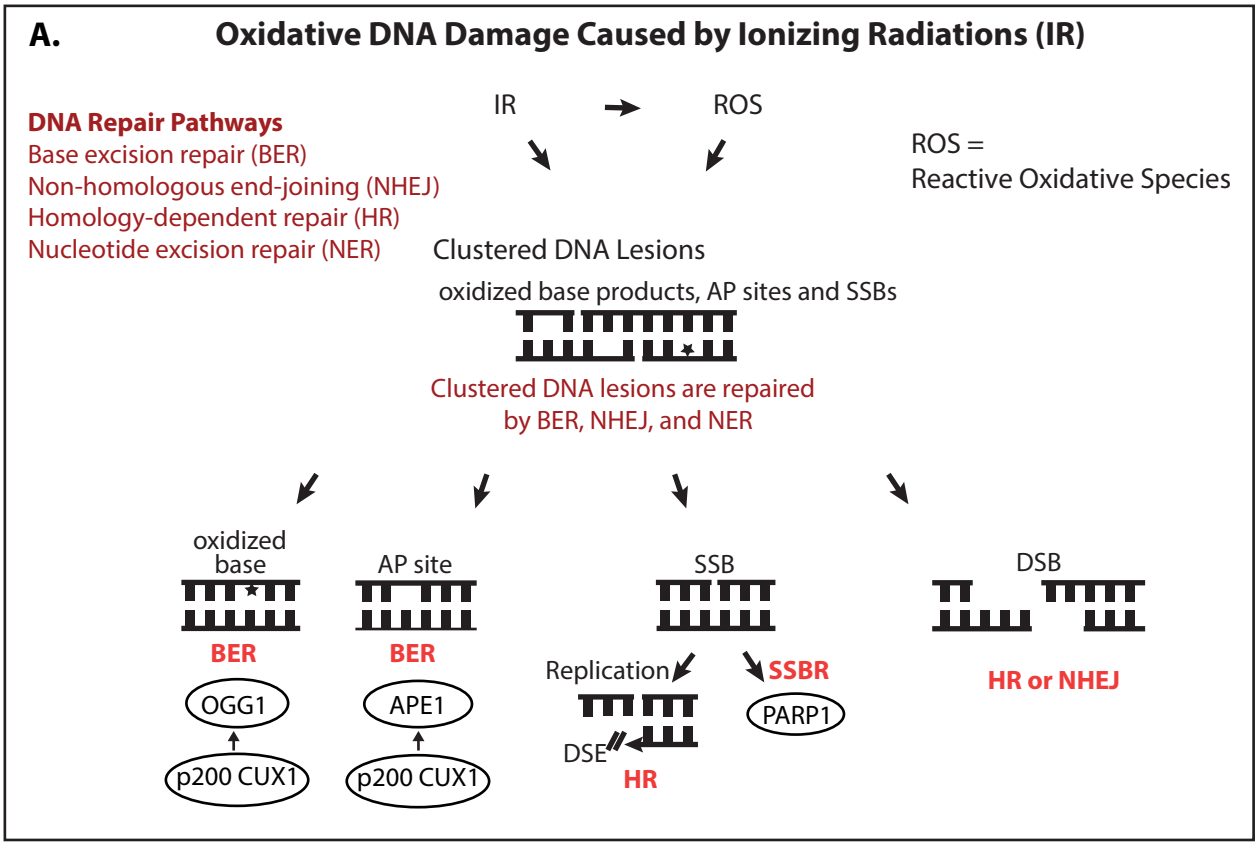
Fig. 6

Figure 7: Proposed Role of CUX1 in the Repair of IR-Induced DNA Damage

A. Oxidative Damage Caused by Ionizing Radiation

Ionizing radiations (IR), through the production of reactive oxidative species, generate a wide variety of DNA lesions including single-strand breaks, abasic sites, and oxidized bases, which are primarily repaired through the base excision repair (BER) pathway. Lesions that interfere with or block DNA replication are toxic and can be lethal if not effectively repaired. Toxic lesions include some oxidized bases such as FapyG, AP sites, single-strand breaks (SSBs) and double-strand breaks (DSBs). DSBs are generated when two SSBs are produced close to each other on adjacent DNA strands (lesion on the right). In addition, replication of a strand containing a single-strand break produces a type of DSB called double-strand end (DSE). DSBs are repaired by homologous recombination (HR) or non-homologous end-joining (NHEJ). SSBs are recognized by PARP1, which then recruits 5' and 3'end-processing enzymes and proteins involved in repair synthesis. Repair of many oxidized bases is initiated by OGG1, while repair of AP sites is initiated by APE1. The p200 CUX1 protein was shown to function as an accessory factor that stimulates OGG1 through its Cut repeats[92 100]. Moreover, unpublished observations indicate that p200 CUX1, via its Cut repeats, can also stimulate APE1[112]. Thus we postulate that CUX1 and in particular the Cut repeats, could represent a therapeutic target: a drug that inhibits the stimulation of OGG1 and APE1 by Cut repeats may be able to sensitize cancer cells to radiotherapy.

B. Isoforms of CUX1. The full-length p200 CUX1 protein is proteolytically processed by cathepsin L to generate the p110 CUX1 isoform. Evolutionarily conserved regions are shown: CUT repeat 1 (CR1), CR2, and CR3, and the CUT homeodomain (HD). Importantly Dr. Nepveu's laboratory created a recombinant protein containing two Cut repeats, which stimulates DNA repair yet is devoid of transcriptional activity[100]. This recombinant protein is useful in measuring solely the effect of CUX1 DNA repair function.


A.
Oxidative DNA Damage Caused by Ionizing Radiations (IR)
DNA Repair Pathways
Base excision repair (BER)
Non-homologous end-joining (NHEJ)
Homology-dependent repair (HR)
Nucleotide excision repair (NER)
IR
ROS
ROS =
Reactive Oxidative Species
Clustered DNA Lesions
oxidized base products, AP sites and SSBs
Clustered DNA lesions are repaired
by BER, NHEJ, and NER
oxidized base
AP site
SSB
DSB
BER
BER
Replication
SSBR
HR or NHEJ
OGG1
APE1
PARP1
p200 CUX1
p200 CUX1
DSE
HR

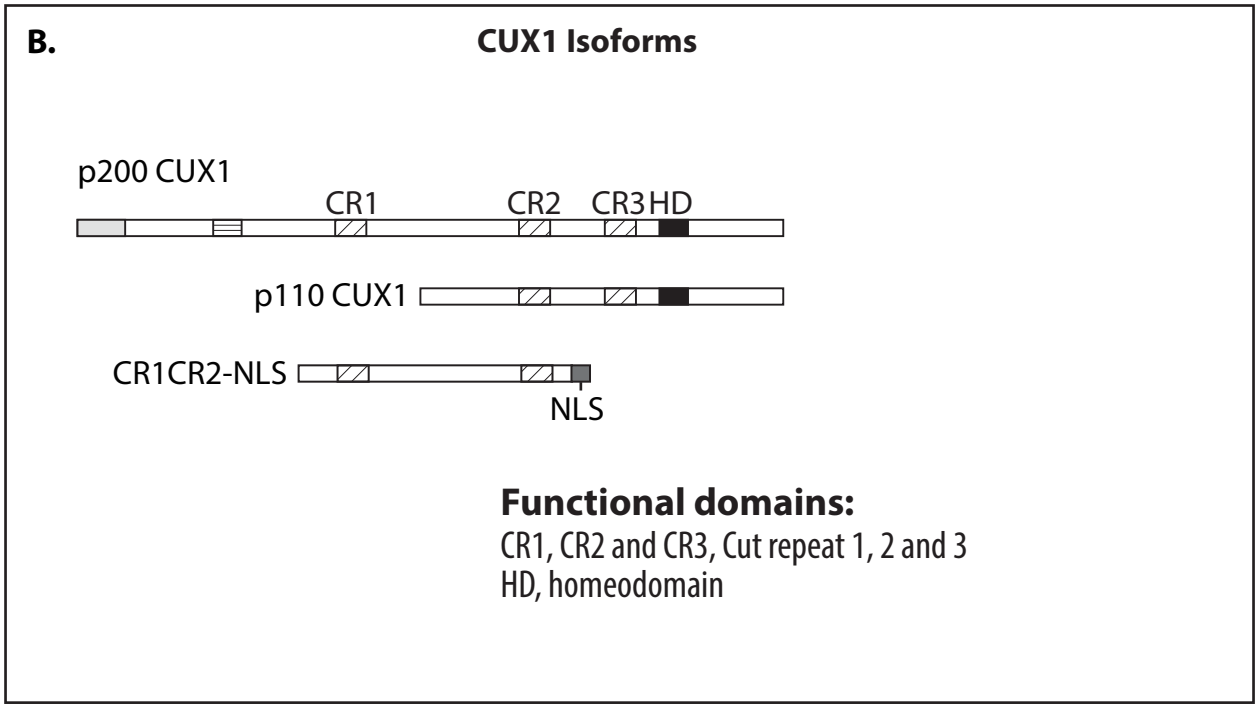

B.
CUX1 Isoforms
p200 CUX1
CR1
CR2
CR3
HD
p110 CUX1
CR1CR2-NLS
NLS
Functional domains:
CR1, CR2 and CR3, Cut repeat 1, 2 and 3
HD, homeodomain

Fig. 7

Figure 8: Methyl-^{14}C Thymidine Incorporation in Hs578T Breast Tumor Cells Treated with Ionizing Radiation

Hs578T breast cancer cells were irradiated (0, 1, 2, and 4 Gy). After being irradiated at the indicated doses, 4000 cells were plated per well in the 96-well Cytostar scintillating plates, in the presence of radioactively-labeled methyl-^{14}C thymidine. Thymidine incorporation was measured for 3 days. Data was normalized to the initial readings on Day 0.

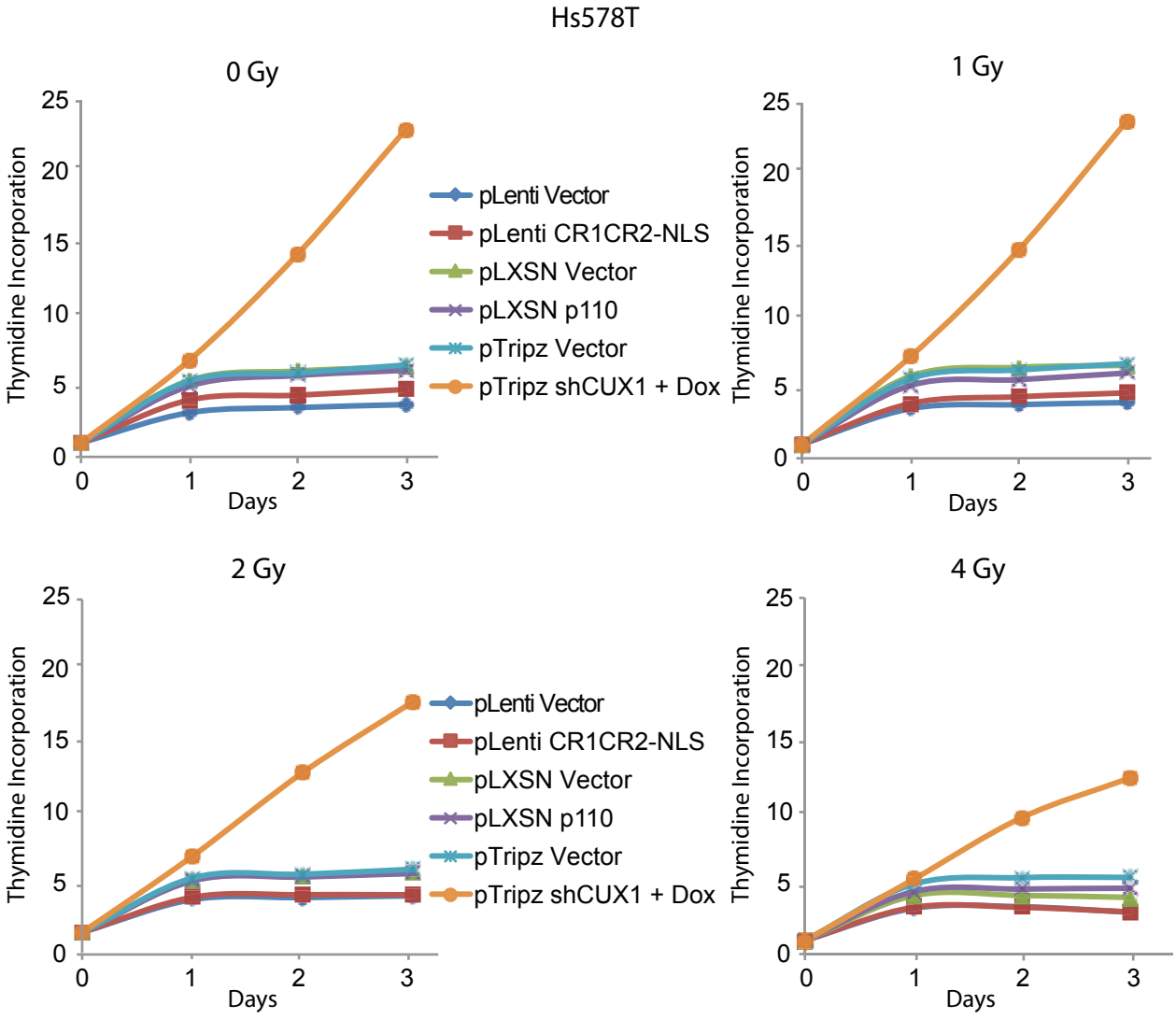
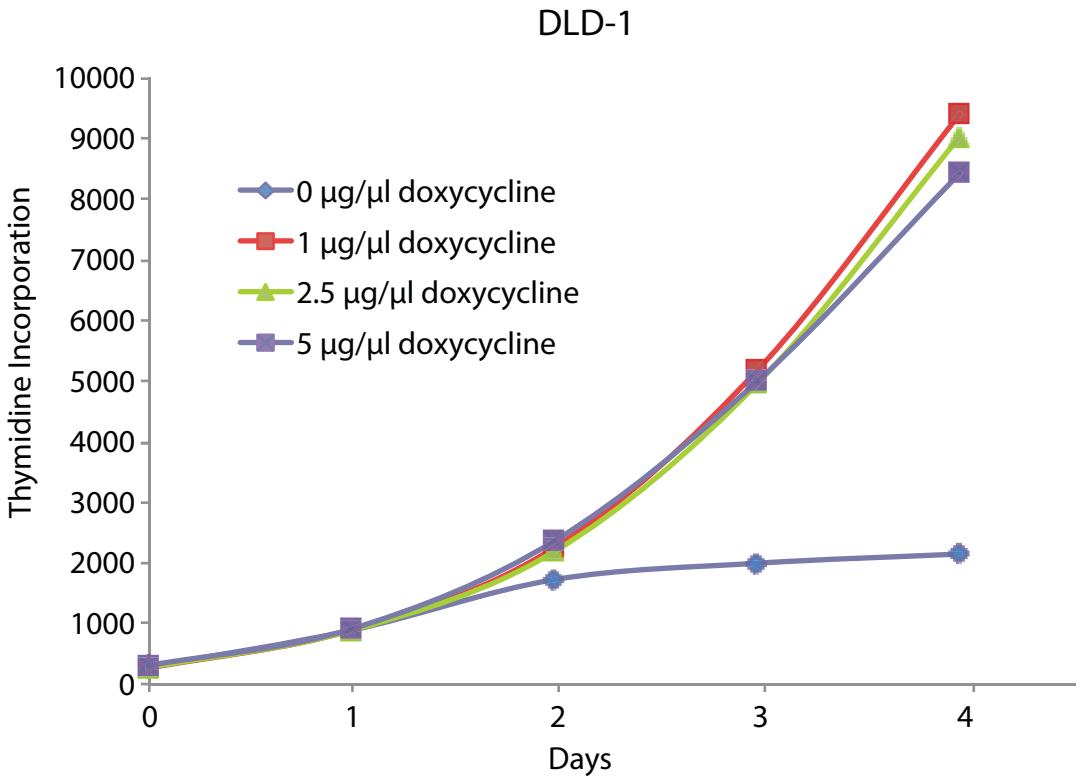

Hs578T
0 Gy
1 Gy
2 Gy
4 Gy
Thymidine Incorporation
Days
0
5
10
15
20
25
0
1
2
3
pLenti Vector
pLenti CR1CR2-NLS
pLXSN Vector
pLXSN p110
pTripz Vector
pTripz shCUX1 + Dox

Fig. 8

Figure 9: Effect of Doxycycline on Methyl-^{14}C Thymidine Incorporation

Hs578T breast and DLD-1 colorectal cancer cells were treated with doxycycline (0, 1, 2.5, and 5 μg/μl). 4000 cells were plated per well in the 96-well Cytostar scintillating plates, in the presence of radioactively-labeled methyl-^{14}C thymidine. Thymidine incorporation was measured for 4 days. Data was normalized to the initial readings on Day 0.


DLD-1
Thymidine Incorporation
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0 µg/µl doxycycline
1 µg/µl doxycycline
2.5 µg/µl doxycycline
5 µg/µl doxycycline
0
1
2
3
4
Days

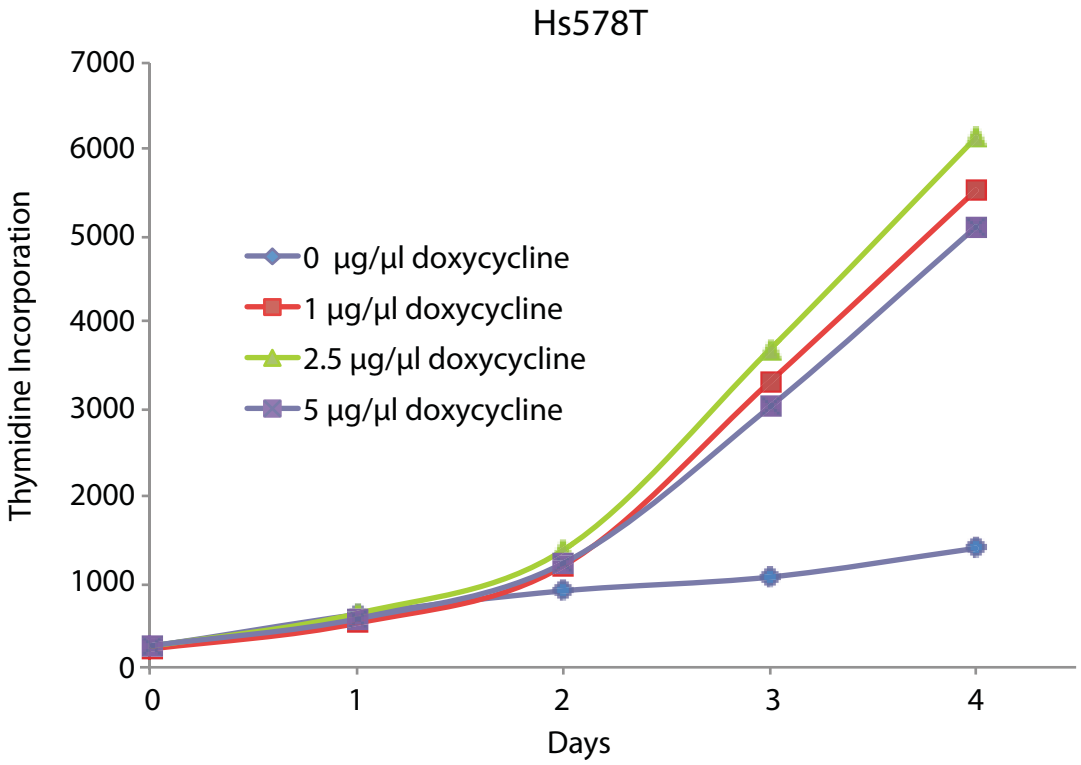
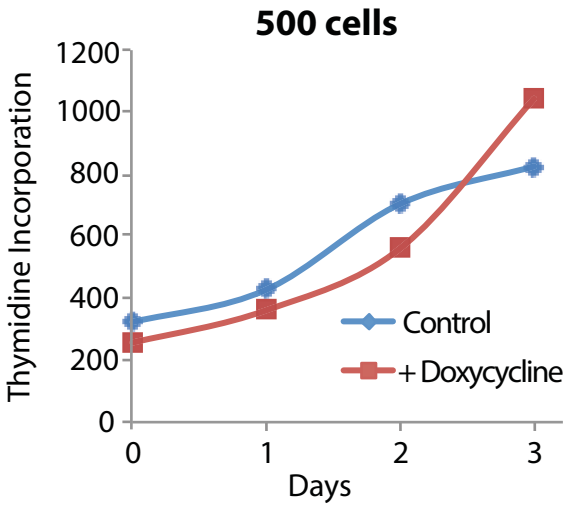
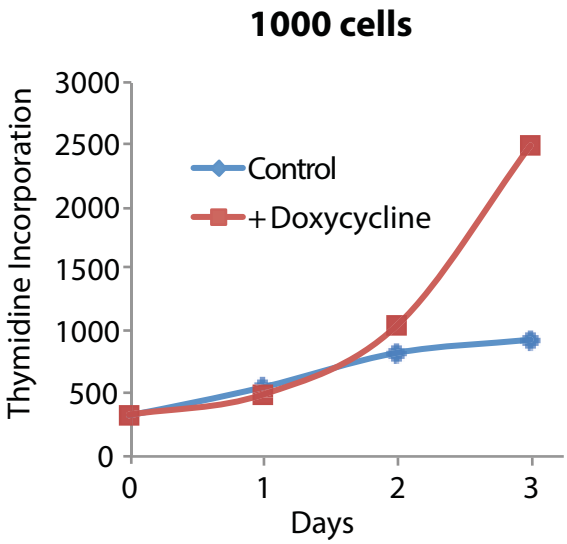

Hs578T
Thymidine Incorporation
7000
6000
5000
4000
3000
2000
1000
0
0 µg/µl doxycycline
1 µg/µl doxycycline
2.5 µg/µl doxycycline
5 µg/µl doxycycline
0
1
2
3
4
Days

Fig. 9

Figure 10: Effect of Doxycycline and Cell Density on Methyl-^{14}C Thymidine Incorporation of Hs578T Breast Tumor Cells

Hs578T breast cancer cells were treated with or without 5µg/µl doxycycline and were plated at different cell densities (500, 1000, 4000 cells) per well in the 96-well Cytostar scintillating plates, in the presence of radioactively-labeled methyl-^{14}C thymidine. Thymidine incorporation was measured for 3 days. Data was normalized to the initial readings on Day 0.


500 cells
Thymidine Incorporation
1200
1000
800
600
400
200
0
0
1
2
3
Days
Control
+ Doxycycline


1000 cells
Thymidine Incorporation
3000
2500
2000
1500
1000
500
0
0
1
2
3
Days
Control
+ Doxycycline

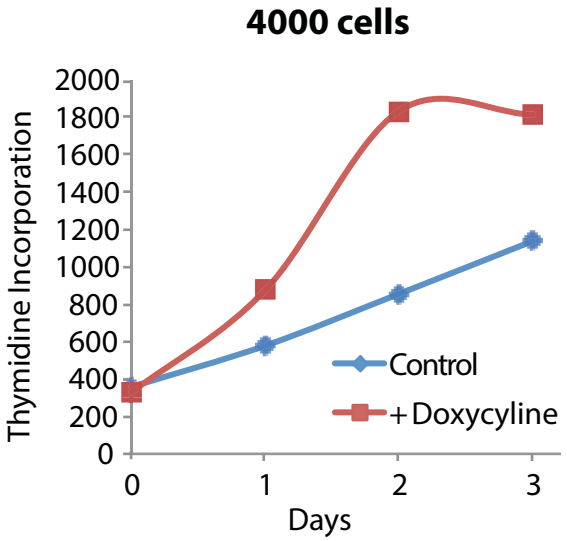

4000 cells
Thymidine Incorporation
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
1
2
3
Days
Control
+ Doxycyline

Fig. 10

Figure 11: Effect of Ionizing Radiation Dose on DNA Damage as Detected in Single Cell Electrophoresis

DLD-1 (A), T98G (B), and Hs578T (C) tumor cells were irradiated at the indicated doses and then submitted to single cell gel electrophoresis either at pH >13 or at pH 10. Single-strand DNA breaks (SSBs), double-strand DNA breaks (DSBs), AP sites and alkaline labile altered bases are detected at pH >13, whereas only SSBs and DSBs are detected at pH 10.
Comet tail moments were scored for at least 50 cells per conditions. Error bars represent standard error.
*p<0.05, **p<0.01, ***p<0.001; Student's t-test.

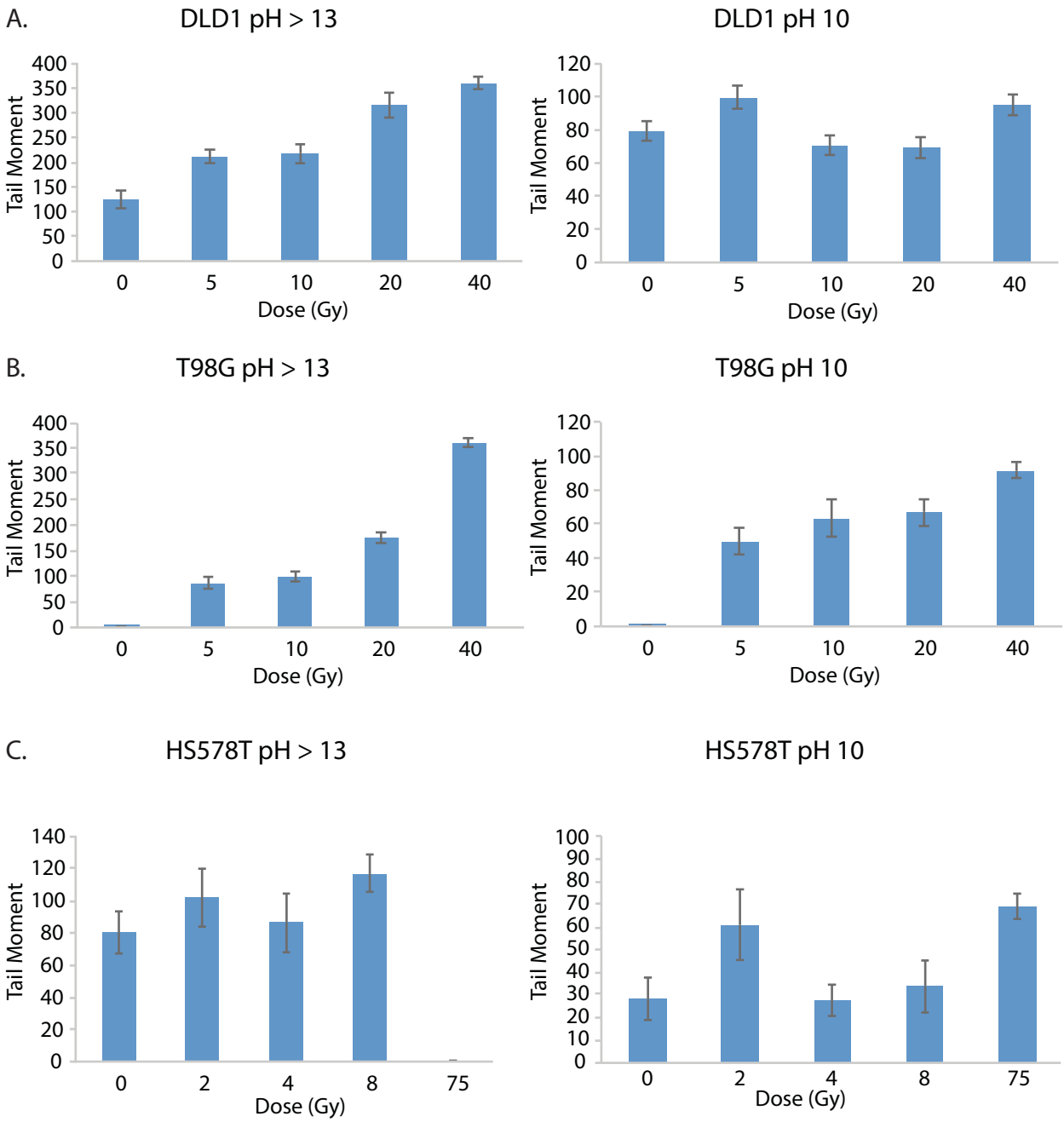

A.
DLD1 pH > 13
Tail Moment
400
350
300
250
200
150
100
50
0
0
5
10
20
40
Dose (Gy)
DLD1 pH 10
Tail Moment
120
100
80
60
40
20
0
0
5
10
20
40
Dose (Gy)
B.
T98G pH > 13
Tail Moment
400
350
300
250
200
150
100
50
0
0
5
10
20
40
Dose (Gy)
T98G pH 10
Tail Moment
120
100
80
60
40
20
0
0
5
10
20
40
Dose (Gy)
C.
HS578T pH > 13
Tail Moment
140
120
100
80
60
40
20
0
0
2
4
8
75
Dose (Gy)
HS578T pH 10
Tail Moment
100
90
80
70
60
50
40
30
20
10
0
0
2
4
8
75
Dose (Gy)

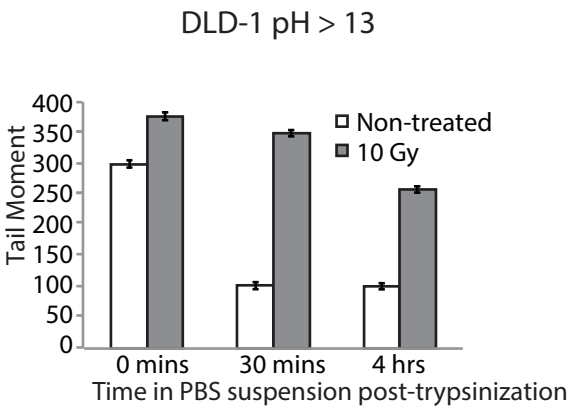
Fig. 11

Figure 12: Optimization of Single Cell Electrophoresis Following Ionizing Radiation

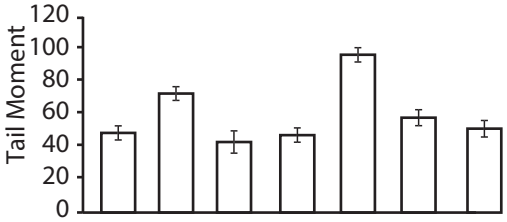
(A) DLD-1 colorectal tumor cells were trypsinized, resuspended in PBS, irradiated at the indicated doses and allowed to recover for the indicated times. Cells that were not irradiated were maintained in PBS for the same periods of time. Cells were then submitted to single cell gel electrophoresis at pH>13.

(B) DLD-1 colorectal tumor cells were irradiated at the indicated doses either in the plate prior to trypsinization, in a tube after trypsinization and resuspension in PBS, or after embedding in agarose directly on the comet assay slide. Comet assays were performed at either pH >13 or pH 10. Comet tail moments were scored for at least 50 cells per conditions. Error bars represent standard error.
*p<0.05, **p<0.01, ***p<0.001; Student's t-test.

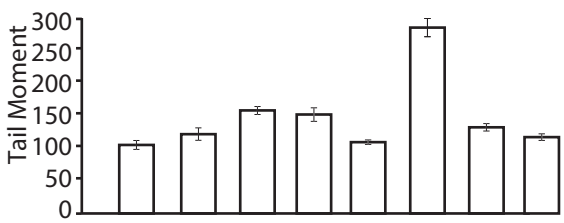
A.



B.



Radiation (Gy)	0	5	5	5	10	10	10
Method	plate	agar	plate	plate	agar	plate	plate
Recovery (h)		0	0	1	0	0	1
Experiment #	1	2	3	4	5	6	7



Radiation (Gy)	0	5	5	5	5	10	10	10
Method	plate	plate	agar	tube	plate	agar	plate	plate
Recovery (h)		0	0	0	1	0	0	1
Experiment #	8	9	10	11	12	13	14	15

Fig. 12

Figure 13. Effect of DNA Staining on Image Quality of the Comet Assay

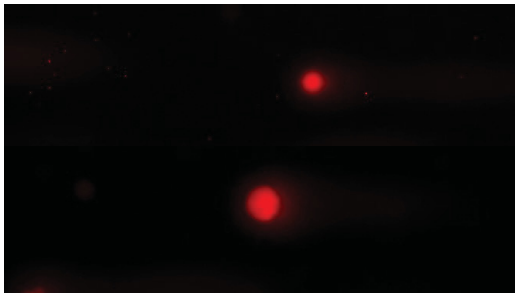
DLD-1 colorectal tumor cells were treated with ionizing radiation at 10 Gy and allowed to recover for 0h or 1h, before single cell gel electrophoresis at pH > 13. Then the agarose-embedded cells were either ethanol-dried (C and G) or left in agarose (A and E), and stained with either SYBR Green (E and G) or propidium iodide (PI, A and C). Images were taken through an Axiovert wide-field microscope.

Comet tail moments were scored for at least 50 cells per conditions. Error bars represent standard error.

*p<0.05, **p<0.01, ***p<0.001; Student's t-test.

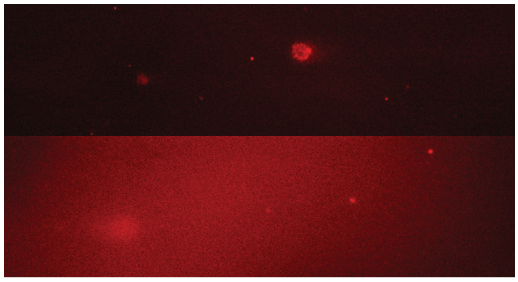
Images taken from Axiovert Wide-field Microscope

A.



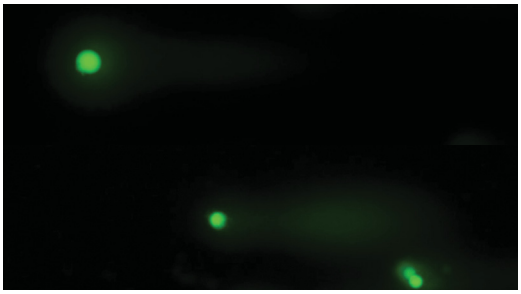
Agarose slides Propidium iodide

C.



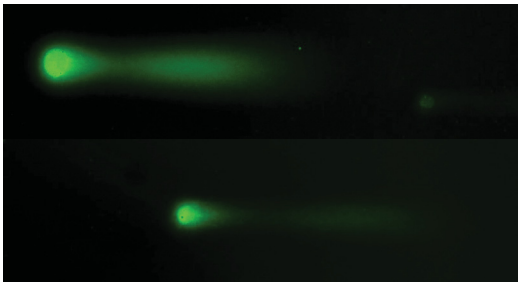
Ethanol dried slides Propidium iodide

E.



Agarose slides SYBR Green

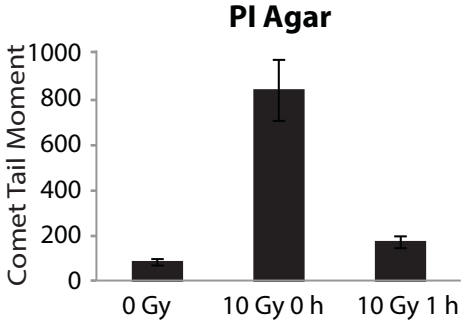
G.



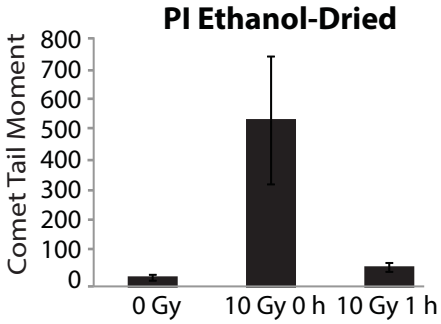
Ethanol dried slides SYBR Green

pH > 13

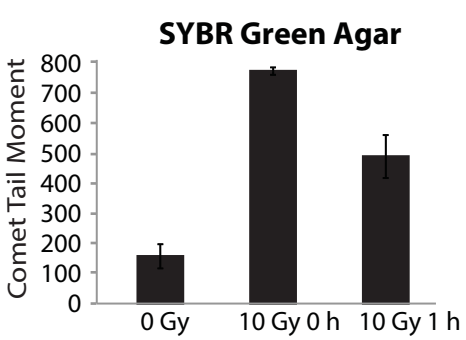
B.



D.



F.



H.

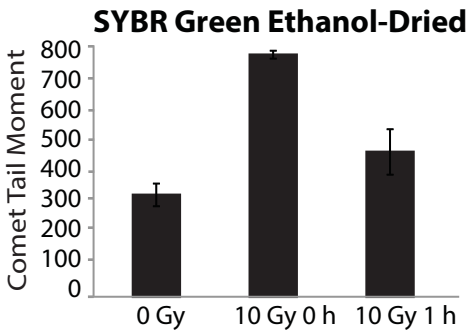


Fig. 13

Figure 14: Effect of p110 CUX1 Overexpression on Clonogenic Efficiency Following Ionizing Radiation.

Hs578T tumor cells were stably infected with retroviruses expressing p110 CUX1-HA or nothing (vector). After being irradiated at the indicated doses, 100 cells were plated in triplicate in 100 mm-plates and incubated for 10 days. Cell colonies were fixed, stained with crystal violet and counted. Error bars represent standard error.
*p<0.05, **p<0.01, ***p<0.001; Student's t-test.

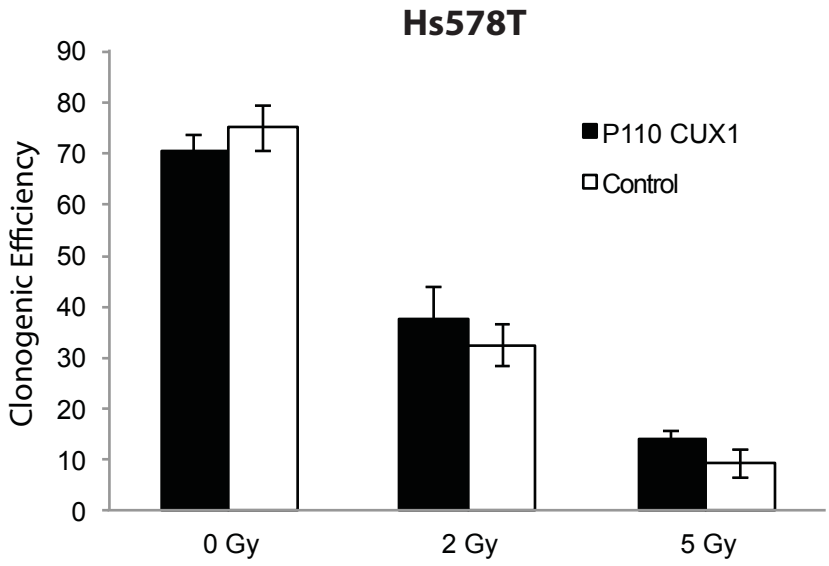

Hs578T
Clonogenic Efficiency
90
80
70
60
50
40
30
20
10
0
P110 CUX1
Control
0 Gy
2 Gy
5 Gy

Fig. 14

Figure 15: Effect of p110 CUX1 Overexpression on Methyl-^{14}C Thymidine Incorporation Following Ionizing Radiation.

DLD-1 (A), MKN-45 (B), and Hs578T (C) cancer cells were stably infected with retroviruses expressing p110 CUX1-HA or nothing (vector). After being irradiated at the indicated doses, 4000 cells were plated per well in the 96-well Cytostar scintillating plates, in the presence of radioactively-labeled methyl-^{14}C thymidine. Thymidine incorporation was measured for 3-5 days. Data was normalized to the initial readings on Day 0.

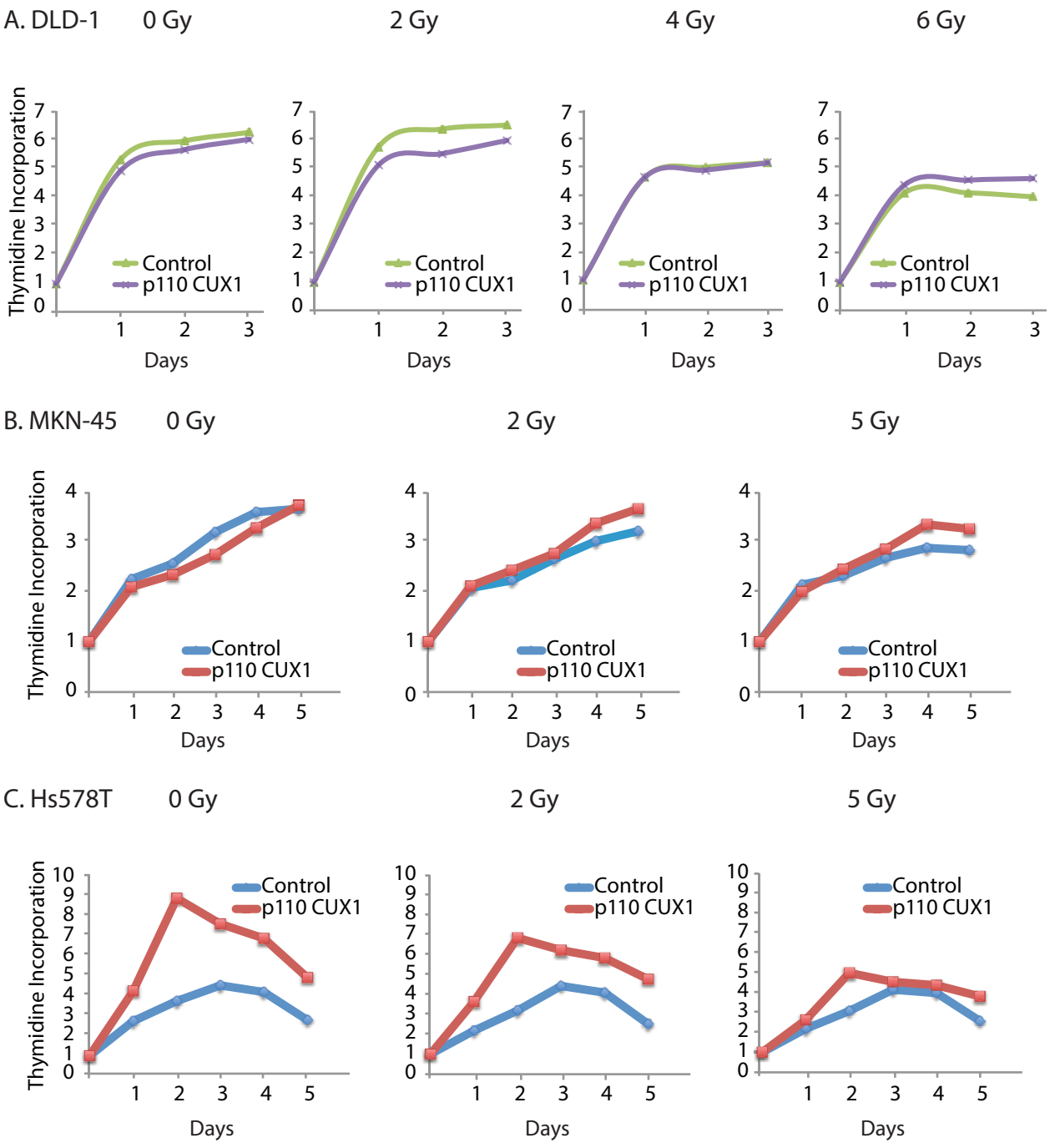

A. DLD-1
0 Gy
2 Gy
4 Gy
6 Gy
Thymidine Incorporation
Control
p110 CUX1
Days
B. MKN-45
0 Gy
2 Gy
5 Gy
C. Hs578T
0 Gy
2 Gy
5 Gy

Fig. 15

Figure 16: Effect of p200 CUX1 Overexpression on Clonogenic Efficiency Following Ionizing Radiation.

T98G (A), U87 (B), DLD-1 (C), and MDA-MB-231 (D) tumor cells were stably infected with retroviruses expressing p200 CUX1-HA or nothing (vector). After being irradiated at the indicated doses, 200 cells were plated in triplicate in 6-well plates and incubated for 10 days. Cell colonies were fixed, stained with crystal violet and counted. The cloning efficiency of unexposed cells was set to 100%. Error bars represent standard error.
*$p<0.05$, **$p<0.01$, ***$p<0.001$; Student's t-test.

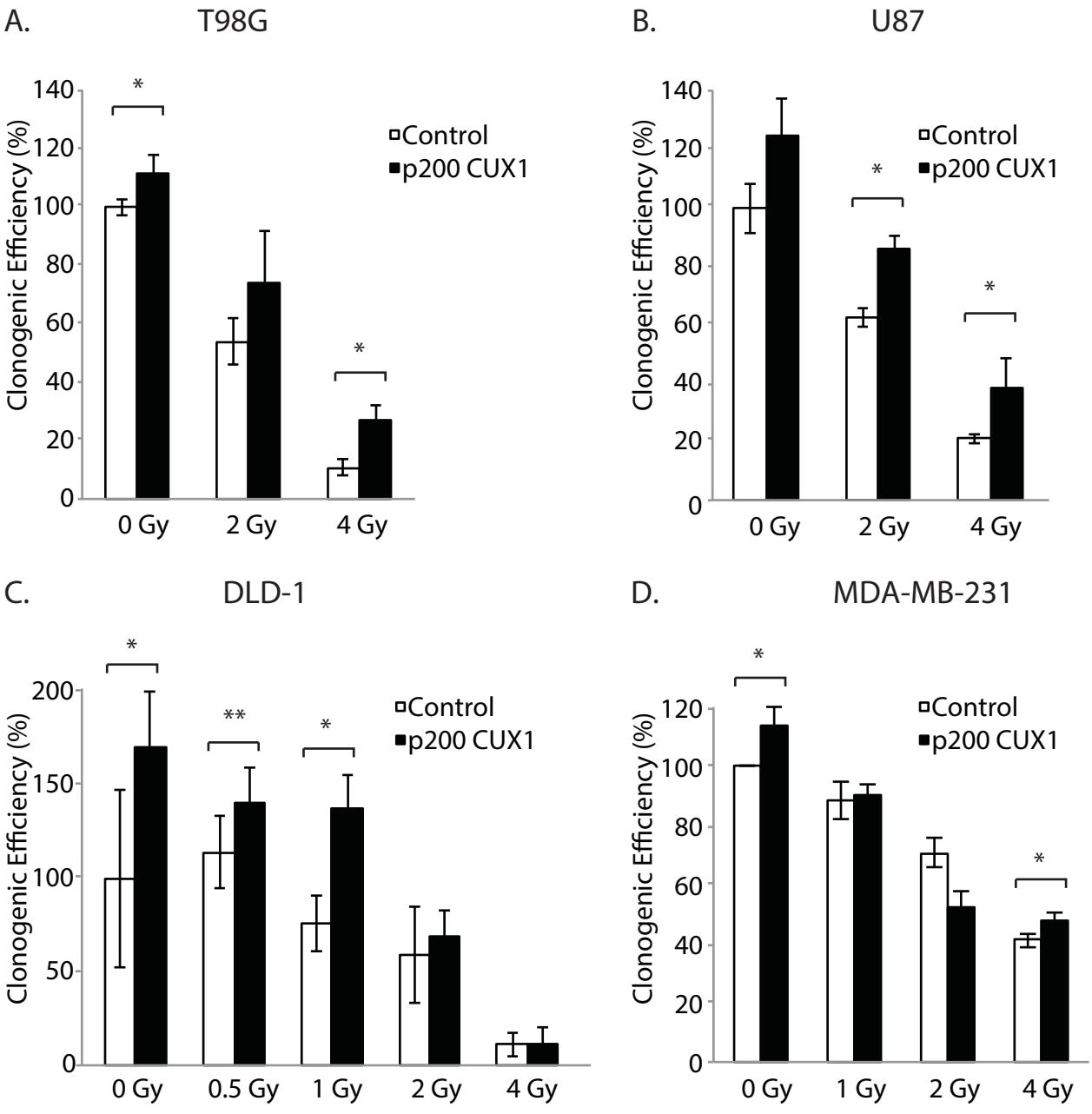

A.
T98G
Clonogenic Efficiency (%)
140
120
100
80
60
40
20
0
*
*
Control
p200 CUX1
0 Gy
2 Gy
4 Gy
B.
U87
Clonogenic Efficiency (%)
140
120
100
80
60
40
20
0
*
*
Control
p200 CUX1
0 Gy
2 Gy
4 Gy
C.
DLD-1
Clonogenic Efficiency (%)
200
150
100
50
0
*
**
*
Control
p200 CUX1
0 Gy
0.5 Gy
1 Gy
2 Gy
4 Gy
D.
MDA-MB-231
Clonogenic Efficiency (%)
120
100
80
60
40
20
0
*
*
Control
p200 CUX1
0 Gy
1 Gy
2 Gy
4 Gy

Fig. 16

Figure 17: CUX1 Protein Expression Across Cancer Cell Lines

Protein extracts of the cancer cell lines A427, A827, A1975, A549, DLD-1, Hs578T, HCT116, U251, T98G, and U87 were prepared and submitted to immunoblotting analysis using anti-CUX1 861 (1:1000) antibody and anti-tubulin (1:1000) antibody.

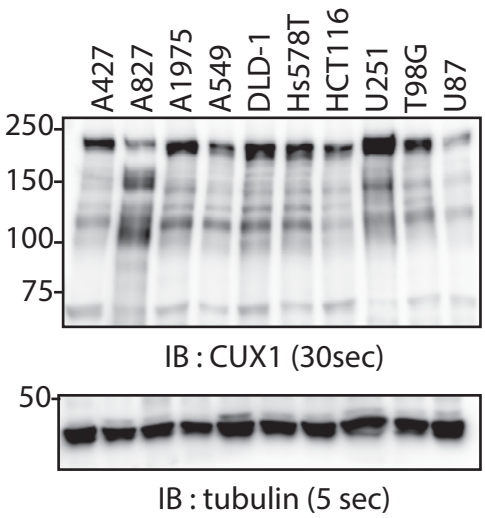

A427
A827
A1975
A549
DLD-1
Hs578T
HCT116
U251
T98G
U87
250
150
100
75
IB : CUX1 (30sec)
50
IB : tubulin (5 sec)

Fig. 17

Figure 18: Effect of OGG1 Knockdown on DNA Synthesis and Clonogenic Efficiency Following Ionizing Radiation.

DLD-1 and U251 tumor cells were transfected with either scrambled or one of three different OGG1 specific siRNA (#s 1, 2, and 4). 96 hours later, protein extracts were prepared. In parallel, cells were submitted to ionizing radiation.

(A) Protein extracts of the cancer cell lines DLD-1 and U251 were submitted to immunoblotting analysis using anti-CUX1 861 (1:1000) antibody, anti-OGG1 (1:1000) and anti-tubulin (1:1000) antibody.

(B) DLD-1 colorectal cancer cells were transfected with OGG1 specific siRNA #2. Four days later, cells were irradiated at 0, 1, 2, and 4 Gy, and 4000 cells were plated per well in the 96-well Cytostar scintillating plate, in the presence of radioactively-labeled methyl-^{14}C thymidine. Thymidine incorporation was measured over the course of 200 hours. Values were normalized to the scramble untreated cell line.

(C) (D) DLD-1 (C) and U251 (D) cancer cells were transfected with the indicated OGG1 specific siRNA. Four days later, cells were irradiated at 0, 1, 2, and 4 Gy, and 200 cells were plated in triplicate in 6-well plates and incubated for 10 days. Cell colonies were fixed, stained with crystal violet and counted. The cloning efficiency of unexposed cells was set to 100%. Error bars represent standard error. *$p<0.05$, **$p<0.01$, ***$p<0.001$; Student's t-test.

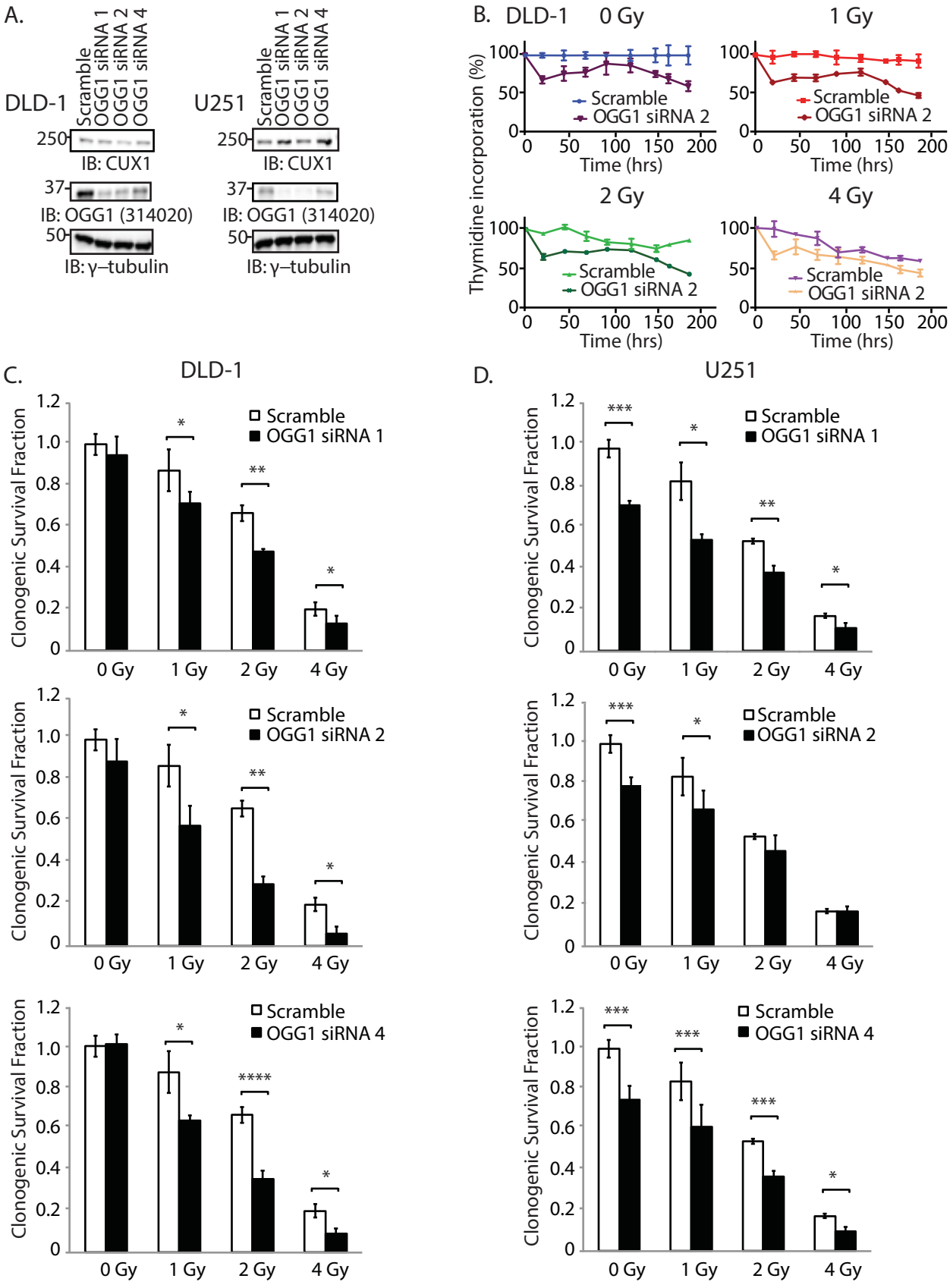

A.
DLD-1
U251
Scramble
OGG1 siRNA 1
OGG1 siRNA 2
OGG1 siRNA 4
250
IB: CUX1
37
IB: OGG1 (314020)
50
IB: γ–tubulin
B. DLD-1
0 Gy
1 Gy
2 Gy
4 Gy
Thymidine incorporation (%)
Time (hrs)
Scramble
OGG1 siRNA 2
C.
D.
Clonogenic Survival Fraction
OGG1 siRNA 1
OGG1 siRNA 4

Fig. 18

Figure 19: Effect of CUX1 Knockdown on Survival after DNA Damage.

T98G, U251, Hs578T, MDA-MB-231, HT29, DLD-1, and HCT116 tumor cells were stably infected with a lentivirus expressing a doxycycline inducible shRNA against CUX1. Cells were treated with doxycycline (+) or not (-) for 4 days prior to radiation treatment. After being submitted to ionizing radiation at the indicated doses, 200 cells were plated in triplicate in 6-well plates and incubated for 10 days. Cell colonies were fixed, stained with crystal violet and counted. The cloning efficiency of unexposed cells was set to 100%. Error bars represent standard error. *$p<0.05$, **$p<0.01$, ***$p<0.001$; Student's t-test.

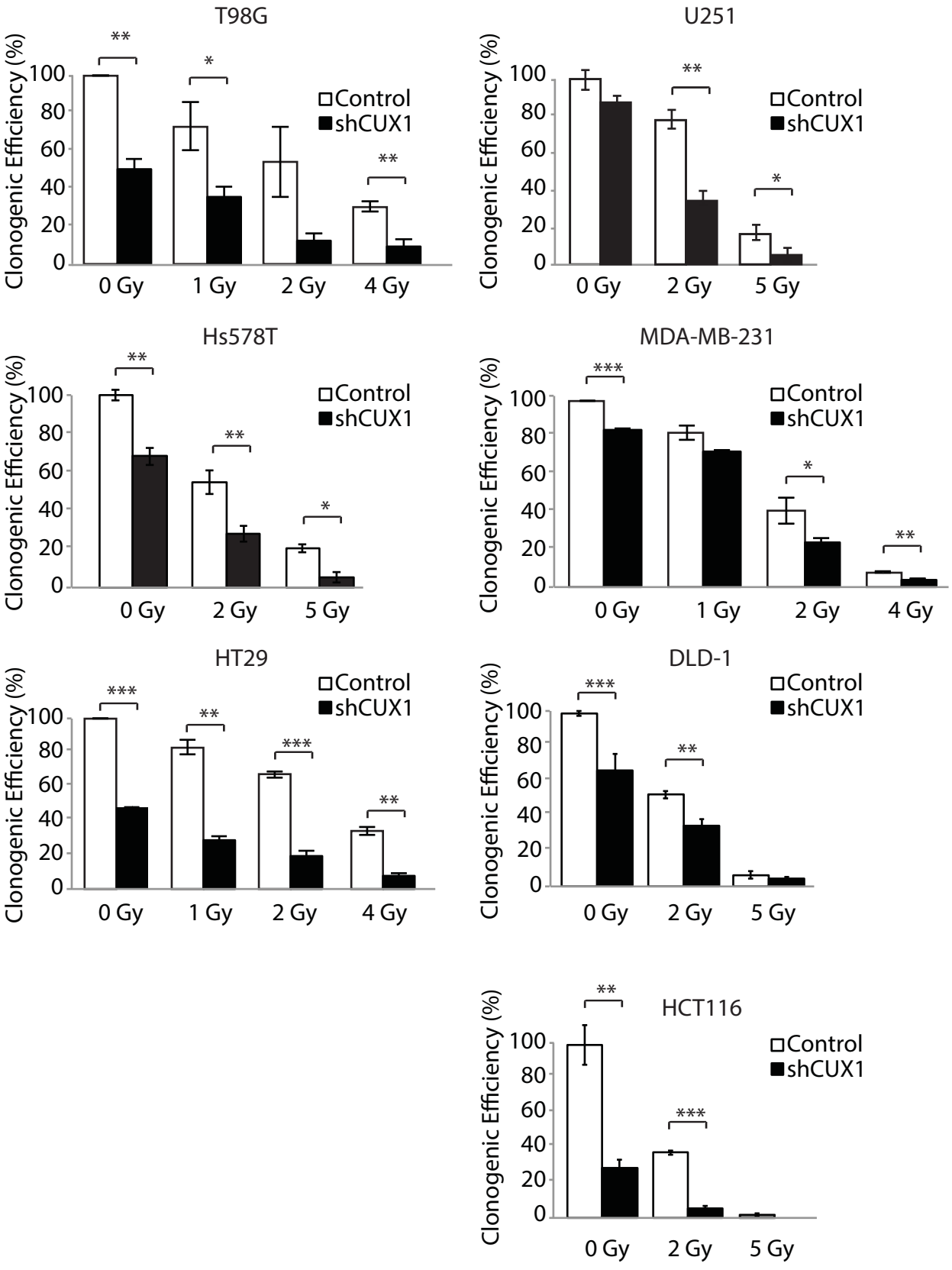
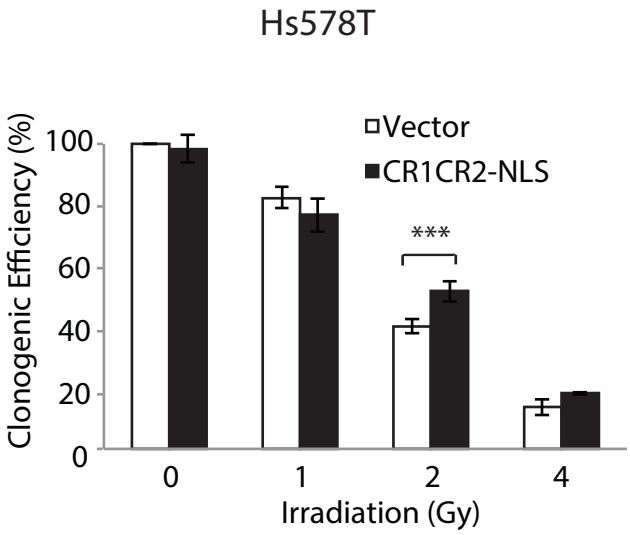

T98G
Clonogenic Efficiency (%)
100
80
60
40
20
0
**
*
**
Control
shCUX1
0 Gy
1 Gy
2 Gy
4 Gy
U251
**
*
0 Gy
2 Gy
5 Gy
Hs578T
**
**
*
0 Gy
2 Gy
5 Gy
MDA-MB-231
***
*
**
0 Gy
1 Gy
2 Gy
4 Gy
HT29
***
**
***
**
0 Gy
1 Gy
2 Gy
4 Gy
DLD-1
***
**
0 Gy
2 Gy
5 Gy
HCT116
**
***
0 Gy
2 Gy
5 Gy

Fig. 19

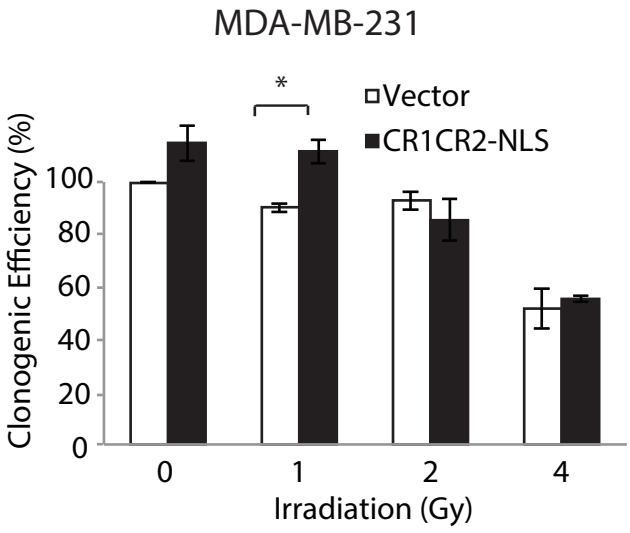
Figure 20: Effect of CR1CR2-NLS Ectopic Expression on Clonogenic Efficiency Following Ionizing Radiation.

Hs578T (A), T98G (B), and MDA-MB-231 (C) tumor cells were stably infected with lentiviruses expressing either nothing (vector) or a recombinant CUX1 protein containing Cut repeats 1 and 2 fused to a nuclear localization signal, CR1CR2-NLS. Following ionizing radiation at 0, 1, 2, and 4 Gy, 200 cells were plated in triplicate in 6-well plates and incubated for 10 days. Cell colonies were fixed, stained with crystal violet and counted. The cloning efficiency of unexposed cells was set to 100%. Error bars represent standard error. *$p<0.05$, **$p<0.01$, ***$p<0.001$; Student's t-test.

A.


Hs578T
Vector
CR1CR2-NLS
***
Clonogenic Efficiency (%)
100
80
60
40
20
0
0
1
2
4
Irradiation (Gy)

B.


MDA-MB-231
*
Vector
CR1CR2-NLS
Clonogenic Efficiency (%)
100
80
60
40
20
0
0
1
2
4
Irradiation (Gy)

C.

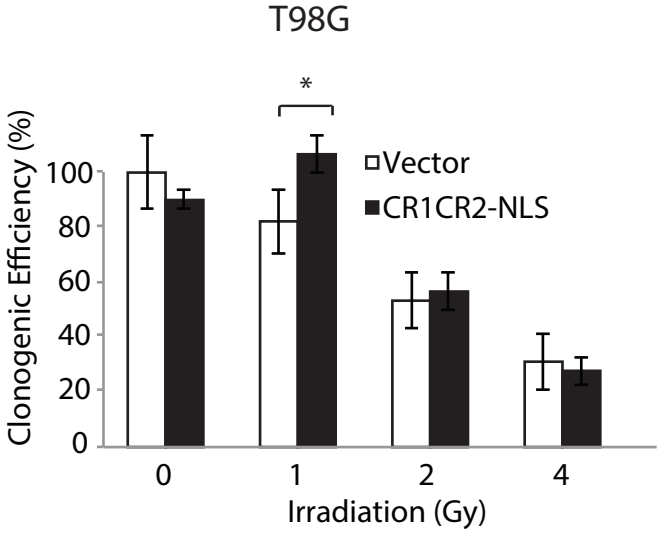

T98G
*
Vector
CR1CR2-NLS
Clonogenic Efficiency (%)
100
80
60
40
20
0
0
1
2
4
Irradiation (Gy)

Fig. 20

Figure 21: Effect of CR1CR2-NLS Ectopic Expression on DNA Synthesis Following Ionizing Radiation.

T98G, Hs578T, MCF7, and DLD-1 cancer cells were stably infected with lentiviruses expressing CR1CR2-NLS or nothing (vector). Following ionizing radiation at 0, 1, 2, 4 or 6 Gy, 4000 cells were plated per well in the 96-well Cytostar scintillating plates, in the presence of radioactively-labeled methyl-^{14}C -thymidine. Thymidine incorporation was measured for 3-5 days.

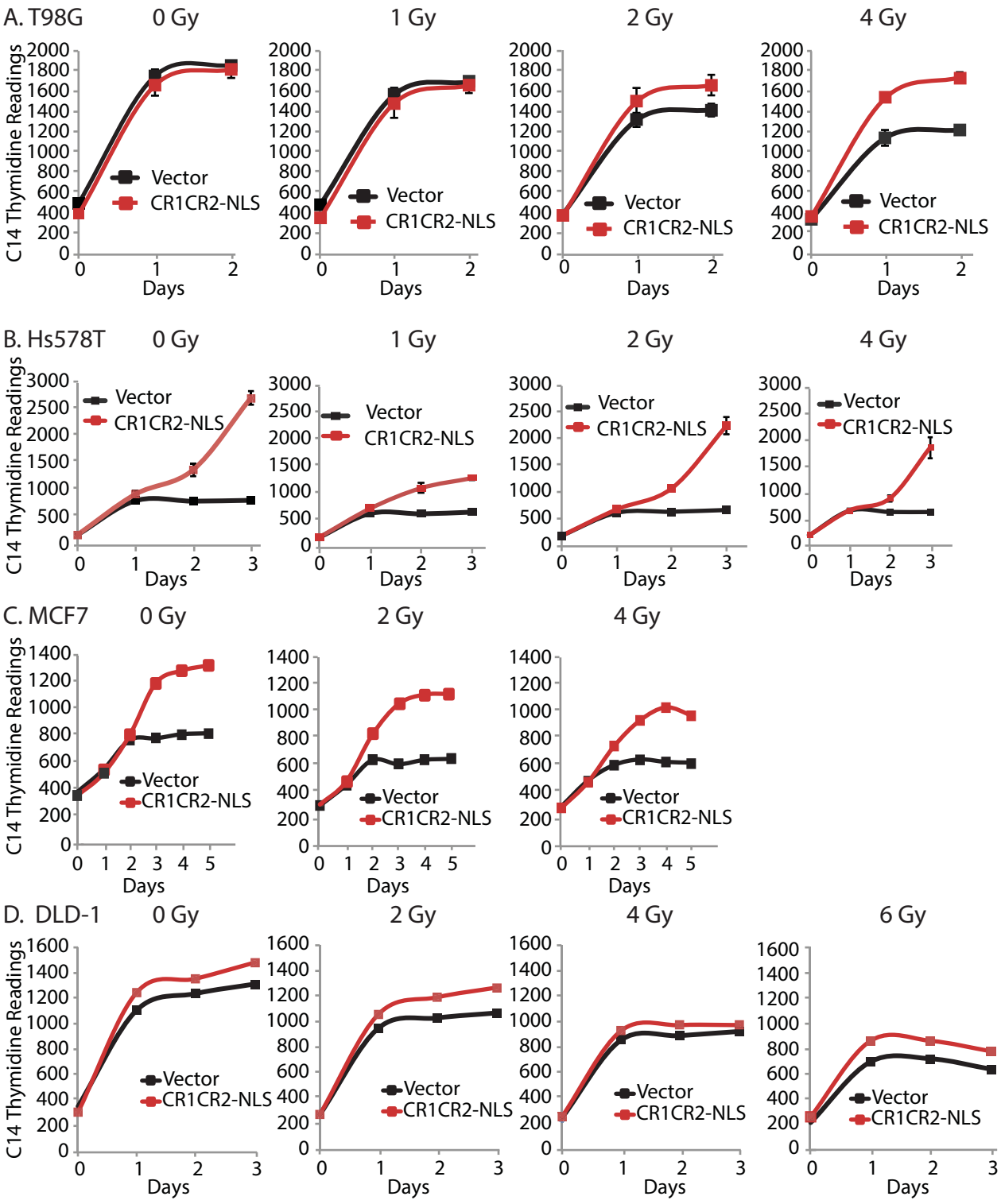

A. T98G
0 Gy
1 Gy
2 Gy
4 Gy
C14 Thymidine Readings
Days
Vector
CR1CR2-NLS
B. Hs578T
0 Gy
1 Gy
2 Gy
4 Gy
C14 Thymidine Readings
Days
Vector
CR1CR2-NLS
C. MCF7
0 Gy
2 Gy
4 Gy
C14 Thymidine Readings
Days
Vector
CR1CR2-NLS
D. DLD-1
0 Gy
2 Gy
4 Gy
6 Gy
C14 Thymidine Readings
Days
Vector
CR1CR2-NLS

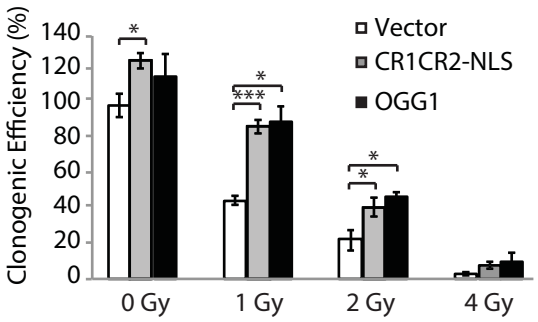
Fig. 21

Figure 22: Effect of CR1CR2-NLS and OGG1 Ectopic Expression on Cell Survival of Tumor and Normal Cell Line Following Ionizing Radiation.

DLD-1 tumor cells (A) and RPE-1 normal retinal pigment epithelial cells (B) were stably infected with lentiviruses expressing CR1CR2-NLS, OGG1 protein, or nothing (vector). Following ionizing radiation at 0, 1, 2, and 4 Gy, 200 cells were plated in triplicate in 6-well plates and incubated for 10 days. Cell colonies were fixed, stained with crystal violet and counted. The cloning efficiency of unexposed cells was set to 100%. Error bars represent standard error.

*$p<0.05$, **$p<0.01$, ***$p<0.001$; Student's t-test.

A. DLD-1



B. RPE-1

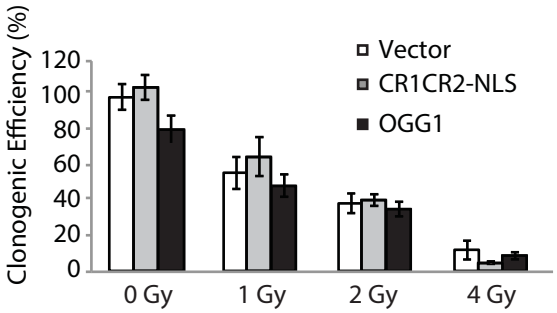


Fig. 22

Figure 23: Single Cell Electrophoresis of T98G Glioblastoma Cells Following Ionizing Radiation.

T98G tumor cells were stably infected with a pTripz vector expressing a doxycycline-inducible shCUX1. Cells were treated or not for four days with doxycycline (control, shCUX1), were irradiated at 10 Gy (or 60 Gy for comet assays at pH 8) and allowed to recover for the indicated times. Then they were submitted to single cell gel electrophoresis in various conditions to quantify DNA damage. The comet slides were then dried with ethanol and stained with SYBR Gold.

(A) Comet assays in alkaline conditions (pH > 13).

(B) Comet assays at pH 10.

(C) Comet assays at pH 10 following treatment with formamidopyrimidine DNA glycosylase (FPG).

(D) Comet assays at pH 8 (60 Gy)

Comet tail moments were scored for at least 50 cells per conditions. Error bars represent standard error.

*p<0.05, **p<0.01, ***p<0.001; Student's t-test.

T98G

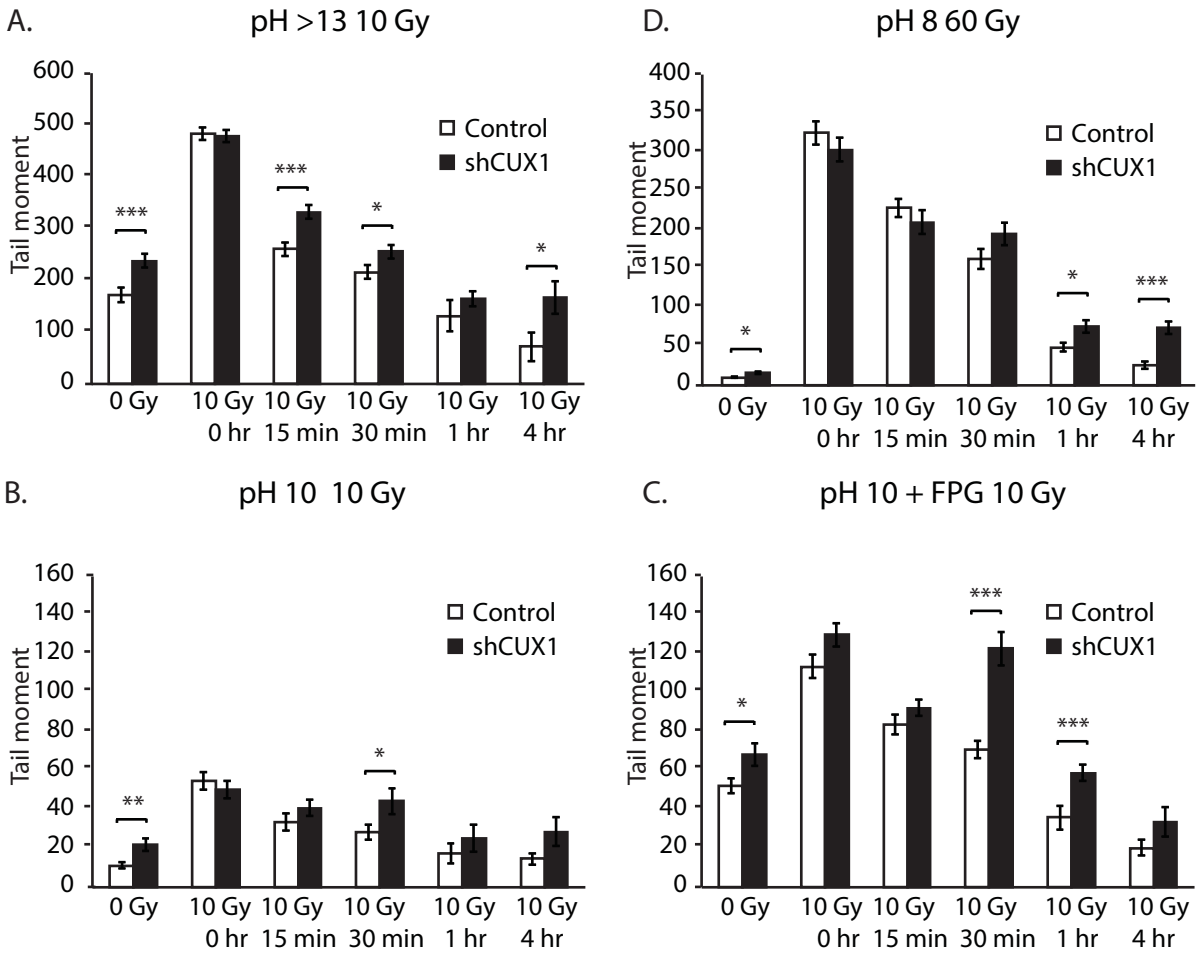

A.
pH >13 10 Gy
Tail moment
Control
shCUX1
0 Gy
10 Gy 0 hr
10 Gy 15 min
10 Gy 30 min
10 Gy 1 hr
10 Gy 4 hr
B.
pH 10 10 Gy
C.
pH 10 + FPG 10 Gy
D.
pH 8 60 Gy

Fig. 23

Figure 24: Single Cell Electrophoresis of DLD-1 Colorectal Cancer Cells Following Ionizing Radiation.

DLD-1 colorectal tumor cells were stably infected with a pTripz vector expressing a doxycycline-inducible shCUX1. Cells were treated or not for four days with doxycycline (control, shCUX1), were submitted to ionizing radiation at 10 Gy, and were allowed to recover for the indicated times. Then they were submitted to single cell gel electrophoresis in various conditions to quantify DNA damage. These cells were then dried with ethanol and stained with SYBR Green.

(A) Comet assays in alkaline conditions (pH > 13).

(B) Comet assays at pH 10.

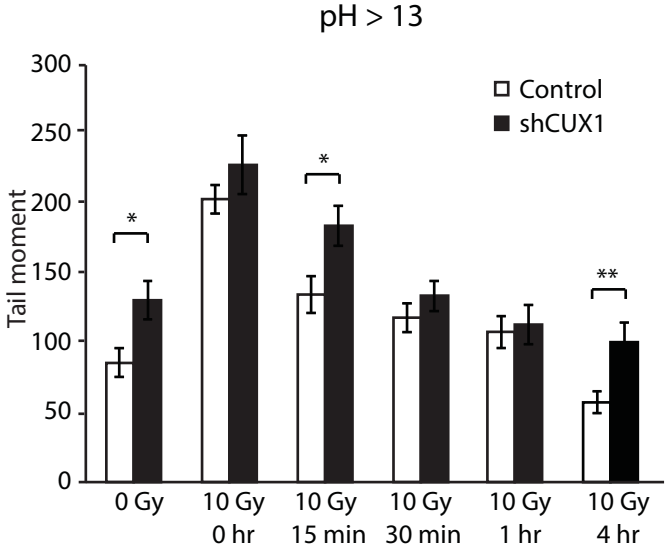
(C) Comet assays at pH 10 following sample treatment with formamidopyrimidine DNA glycosylase (FPG).

Comet tail moments were scored for at least 50 cells per conditions. Error bars represent standard error.

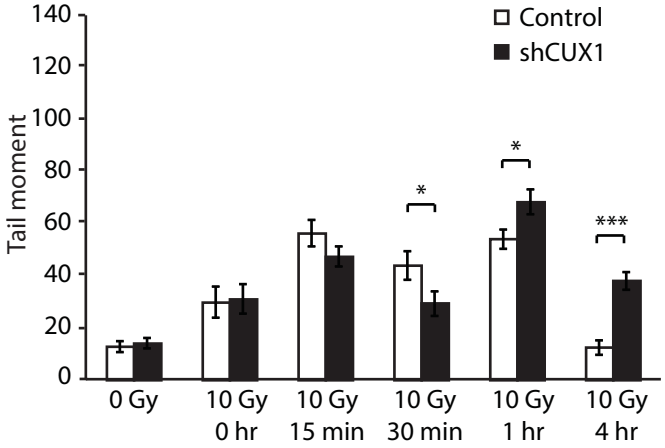
*p<0.05, **p<0.01, ***p<0.001; Student's t-test.

DLD-1

A.



B.



C.

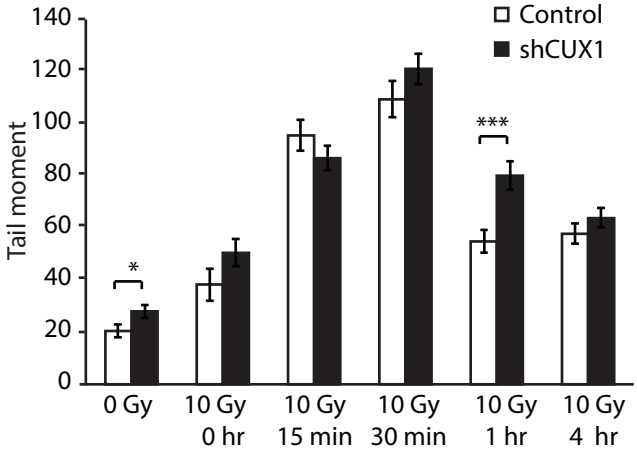


Fig. 24

Figure 25: Effect of CUX1 Knockdown and Overexpression on Clonogenic Survival of Cells Following Treatment with the TH588c MTH1 Inhibitor.

(A-C) DLD-1, Hs578T, and MDA-MB-231 tumor cells were stably infected with a lentivirus expressing a doxycycline inducible shRNA against CUX1. Cells were treated with doxycycline or not for 4 days (control, shCUX1).
(D-G) DLD-1, HCT116, Hs578T, and T98G tumor cells were stably infected with retroviruses expressing p200 CUX1-HA or nothing (vector).
(H-I) DLD-1 and HCT116 cancer cells were stably infected with lentiviruses expressing recombinant CUX1 protein CR1CR2-NLS or nothing (vector).
(J) DKO4 cells were stably infected with lentiviruses expressing recombinant CUX1 protein CR1CR2-NLS or nothing (vector).
(A-L) Cells were treated with or without TH588C at the indicated concentrations. 200 cells were plated in triplicate in 6-well plates and incubated for 10 days. Cell colonies were fixed, stained with crystal violet and counted. The cloning efficiency of unexposed cells was set to 100%. Error bars represent standard error.
*p<0.05, **p<0.01, ***p<0.001; Student's t-test.

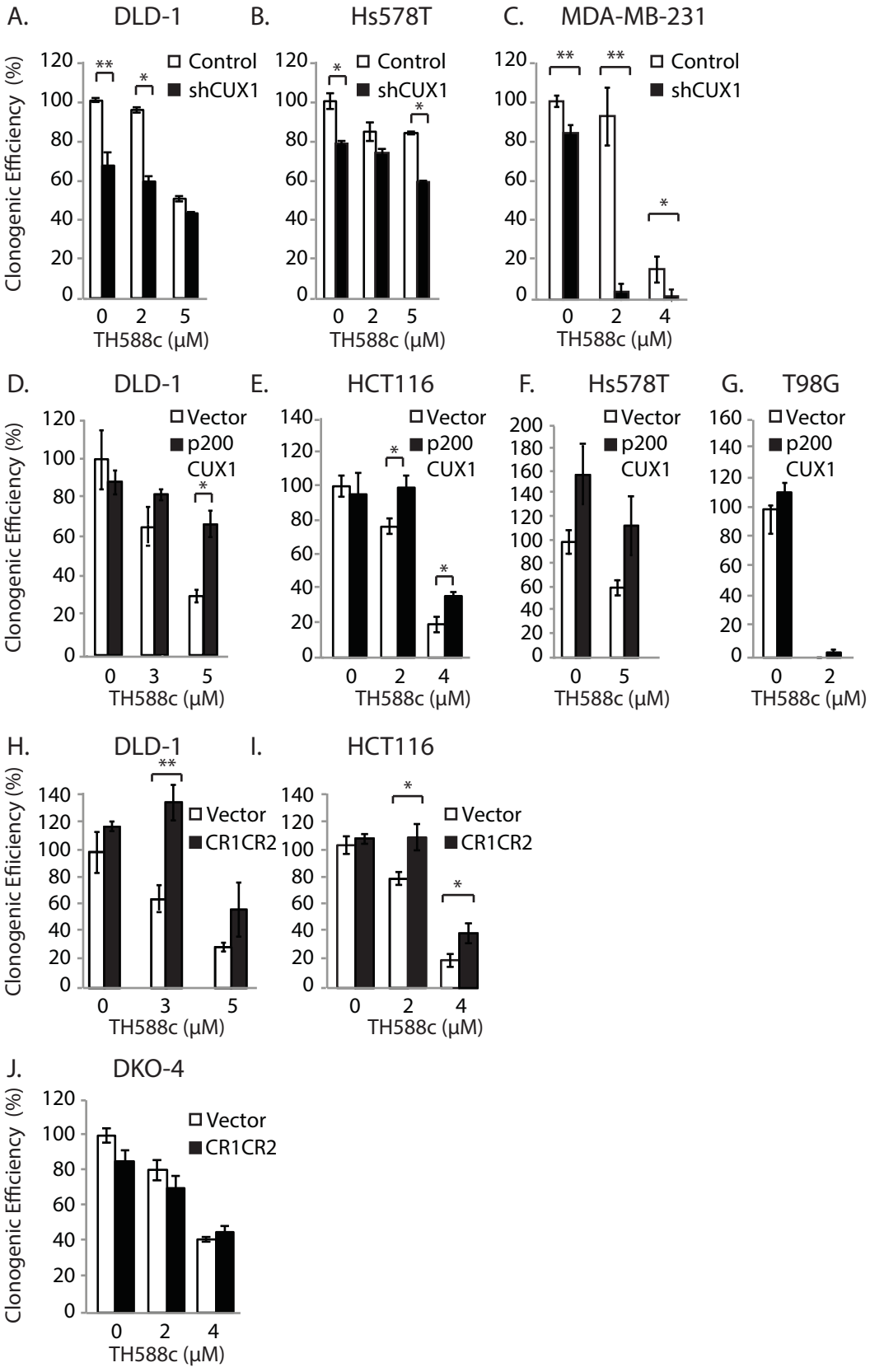

A.
DLD-1
B.
Hs578T
C.
MDA-MB-231
Clonogenic Efficiency (%)
Control
shCUX1
TH588c (μM)
D.
DLD-1
E.
HCT116
F.
Hs578T
G.
T98G
Vector
p200 CUX1
H.
DLD-1
I.
HCT116
Clonogenic Efficiency (%)
Vector
CR1CR2
TH588c (μM)
J.
DKO-4
Clonogenic Efficiency (%)
Vector
CR1CR2
TH588c (μM)

Fig. 25

Figure 26. Immunoblotting Analysis of CUX1, CR1CR2-NLS and OGG1 Expression.

Protein extracts of the indicated cancer cell lines were prepared and submitted to immunoblotting analysis using anti-CUX1 861 (1:1000) antibody, anti-HA antibody, anti-OGG1 antibody, and/or anti-tubulin (1:1000) antibody. Results were organized as follows:

A. Doxycycline-induced CUX1 knockdown
B. CUX1 p200 overexpression
C. CUX1 CR1CR2-NLS overexpression
D. OGG1 overexpression

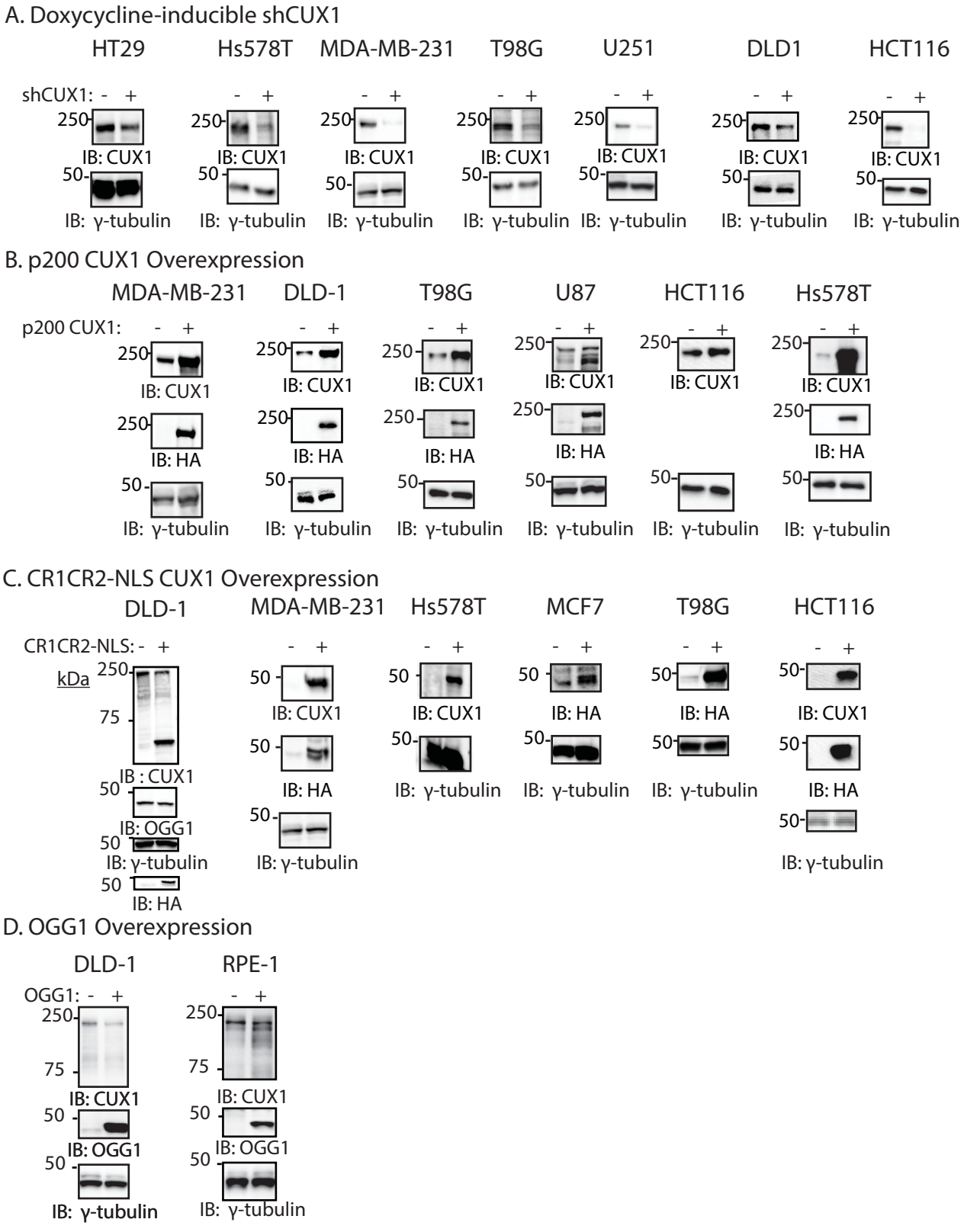

A. Doxycycline-inducible shCUX1
HT29
Hs578T
MDA-MB-231
T98G
U251
DLD1
HCT116
shCUX1: - +
250
IB: CUX1
50
IB: γ-tubulin
B. p200 CUX1 Overexpression
MDA-MB-231
DLD-1
T98G
U87
HCT116
Hs578T
p200 CUX1: - +
IB: HA
C. CR1CR2-NLS CUX1 Overexpression
DLD-1
MDA-MB-231
Hs578T
MCF7
T98G
HCT116
CR1CR2-NLS: - +
kDa
75
IB : CUX1
IB: OGG1
D. OGG1 Overexpression
RPE-1
OGG1: - +

Fig. 26

Discussion

Doxycycline Cannot Be Used in the Methyl-^{14}C Thymidine Incorporation Assays

Evident in the results (**Figs. 8-10**), doxycycline creates an artifact in the methyl-^{14}C thymidine incorporation assay. Determining this allowed us to conclude the necessity to use another assay to investigate the effect of CUX1 knockdown. If we were to use the methyl-^{14}C thymidine incorporation assay to evaluate response of cancer cells to CUX1 knockdown, we would thus use the siRNA transfection method, to eliminate the need for doxycycline.

The Comet Assay was Optimized to Observe DNA Repair in Response to Irradiation

Single-cell gel electrophoresis (comet) assays are commonly used to measure DNA repair in cells. They can be performed at different pH to measure specific types of DNA damage (Electrophoresis at pH 14 measures a wide range of damage, from alkylated bases to oxidative damage to double and single- stranded breaks, pH 10 measures single- and double- stranded breaks, pH 10 with prior treatment of cells with a DNA glycosylase like FPG or OGG1 reveals the presence of oxidized bases in addition to single-strand and double-strand DNA breaks, and pH 8, according to Collins[101], measures solely double-stranded DNA breaks). However, when attempting to measure DNA repair in cancer cells that were irradiated, a few challenges presented themselves.

First, we observed that certain cell lines, DLD-1 and Hs578T, had high levels of endogenous DNA damage (**Fig. 11 A, C**), compared to T98G (**Fig. 11 B**). This is in line with the idea that cell lines carrying a RAS mutant suffer greater DNA damage due to the accumulation of ROS. However, this endogenous damage made it harder to see a difference in DNA damage in response to low doses of irradiation. Thus we opted for treatment at 10 Gy when performing comet assays in alkaline conditions, and at 40-60 Gy when performing comet assays at neutral pH.

In addition, we found that DNA damage due to IR observed in the comet assay was repaired quickly in comparison with that due to H_2O_2. We realized that manipulation of samples between irradiation and embedding into agarose slides lasted around 15 minutes, apparently giving cells ample time to repair IR-induced DNA damage (**Fig. 12**). We conclude the necessity

of a more effective IR "No Recovery" time-point, and established that cells must be embedded into agarose prior to irradiation to produce a more realistic IR "No Recovery" time-point.

Finally, due to accessibility and technical issues, we had to adapt to using a wide-field microscope to capture images of treated cells. Here we observed that our usual propidium iodide stain would yield higher background than with a confocal microscope, which was only exacerbated when the slides were ethanol-dried (**Fig. 13**). Our results lead us to the conclusion that SYBR Green and Gold staining on ethanol-dried slides is preferable, yielding high specificity for DNA binding and very low background.

CUX1 Promotes Radio-Resistance in Cancer Cells through a Direct Role in DNA Repair, in addition to its Transcriptional Function

As I have mentioned in the introduction, one of the major causes of death in cancer patients is due to resistance of tumor cells to radiotherapy. While the mechanism underlying tumor radio-resistance is unclear, we believe that one mechanism of radio-resistance involves the ability of certain cancer cells to more efficiently repair oxidative DNA damage, as a result of their adaptation to their greater metabolic activity and higher production of ROS. Indeed, studies have shown that tumors carrying a mutated *RAS*, which accelerates metabolic activity and the production of ROS, resist more to IR[119]. Importantly, p200 CUX1 knockdown is synthetic lethal with mutated *RAS*. In addition, we have seen that knockdown of p200 CUX1 leads to inefficient repair, shown in increased sensitivity of cancer cells to H_2O_2[120], while overexpression of CUX1 confers resistance to IR (**Figs. 16, 20, and 22**). The aim of my study was to investigate the role of the DNA repair function of CUX1 in promoting resistance to ionizing radiation in cancer cells.

As I have mentioned earlier, there are two main CUX1 protein isoforms: a full-length isoform that functions as an accessory factor in base excision repair[92], and a shorter isoform that functions as a transcription factor[89 90]. Our lab previously established that p110 CUX1 promotes proliferation of cancer cells and activates expression of many genes involved in DNA damage response, including the ATM, ATR, CHK1 and CHK2 checkpoint kinases[96]. Additionally, p110 CUX1 was shown to protect cells against apoptosis induced by tumour necrosis factor-related apoptosis-inducing ligand, TRAIL[84]. I have further confirmed that overexpression of p110 CUX1 confers modest radio-resistance to cells through both clonogenic and methyl-^{14}C thymidine incorporation assays (**Figs. 14 and 15**). In addition, overexpression of full-length

p200 CUX1 significantly increases survival of certain cell lines following irradiation (**Fig. 16**), albeit to a different extent, I believe, depending on their level of endogenous CUX1 expression. For example, we see a more significant effect of p200 CUX1 overexpression in U87 cells, which shows the lowest endogenous p200 CUX1 expression in an immunoblot carried on several parental cancer cell lines (**Fig. 17**). Overexpressing CUX1 full length protein isoform p200 does not confer increased survival in certain cell lines, most likely because these cell lines already express enough CUX1 in order to survive, and overexpressing CUX1 becomes useless when factors downstream of CUX1 are not sufficiently upregulated.

Dr. Nepveu's lab has consistently seen that RNAi knockdown or genetic inactivation of CUX1 reduces ATM expression and negatively impacts protective responses mediated by ATM following exposure to ionizing radiation[94]. These results provided compelling evidence that adequate basal DNA damage response protein levels depend on CUX1 transcriptional regulation, and must be in place prior to sustaining DNA damage such that cells can respond efficiently to mutagenic insult[94]. Consequently, CUX1 knockdown leads to accumulation of oxidative DNA damage, demonstrating that CUX1 is involved in base excision repair and is especially implicated in cancer cells that produce more ROS. Importantly, we were able to ectopically express a recombinant protein containing only two CUT repeat domains with a nuclear localization signal, CR1CR2-NLS, that had previously been shown to be devoid of transcriptional potential[92 100]. We do see that this recombinant protein is rapidly recruited to DNA damage (unpublished observation), accelerates DNA repair and increases resistance to ionizing radiation. These findings indicate that the DNA repair function of CUX1 contributes to the resistance of cancer cells to radiotherapy, and therefore validate CUX1 as a potential therapeutic target for cancer, as inhibiting CUX1 stimulation of BER enzymes would sensitize cancer cells to radiotherapy.

Radio-Sensitivity of Cancer Cells Caused by Decreased OGG1 Expression Validates OGG1 and CUX1 as Therapeutic Targets

Since CUX1 was shown to stimulate OGG1, we have attempted to verify whether OGG1 may play a role in promoting radio-resistance. However, explaining that CUX1 promotes radio-resistance through stimulation of OGG1 was initially challenged by Dr. Susan Wallace's findings that OGG1 overexpression sensitized cancer cells to irradiation in TK6—normal cells[121 122]. We thus sought to observe effect of reduced OGG1 expression on radio-sensitivity of cancer

cells, to validate the role of CUX1 as an accessory factor to BER in radio-resistance of cancer cells. OGG1 knockdown by the two distinct siRNA sensitized both DLD-1 and U251 cancer cells to ionizing radiation (**Fig. 18**). These results demonstrate that OGG1 plays an important role in reducing cytotoxic effects of ionizing radiation in cancer cells.

Absence of CUX1 Sensitizes Cancer Cells to Irradiation, especially those Carrying a Mutant *RAS*

Across different cancer cell lines, CUX1 knockdown sensitizes cancer cells to irradiation (**Fig. 19**). Importantly, CUX1 knockdown alone also affects cancer cells, specifically those carrying an activating mutation in a *RAS* gene, *HRAS* or *KRAS*. Cells that express an activated RAS oncogene produce an excess of reactive oxygen species (ROS) that cause oxidative DNA damage[92 100]. In order to adapt to this damage, they might upregulate the pathway that repairs oxidative DNA damage[92]. We believe that CUX1 is involved in repair of this damage, and as such, CUX1 knockdown prevents efficient repair of oxidative DNA damage induced both by mutant *RAS* and ionizing radiation. For example, DLD-1 cells carry a *KRAS* oncogene and their survival is significantly affected prior to and after irradiation. In support of this notion, among the synthetic lethal interactions with *KRAS* discovered in the genome-wide RNAi screen conducted by the Elledge group were 5 genes that encode proteins involved in base excision repair: *NEIL2*, *CUX1*, *XRCC1*, *Pol beta*, and *LIG3*.[11]

Cut Repeats and OGG1 are Sufficient to Confer Resistance to Radiotherapy in Cancer Cells

Through clonogenics and thymidine incorporation assays, we have confirmed that ectopic expression of the CUX1 Cut repeat domains (CR1CR2-NLS) as well as OGG1 confer radio-resistance to cancer cells (**Figs 20-22**). Importantly, this recombinant CUT domain protein does not function as a transcriptional activator and does not stimulate expression of genes involved in base excision repair yet is able to stimulate OGG1 *in vitro* and in cells[92 100]. Therefore, radio-protection conferred by the CR1CR2-NLS recombinant protein must result from its role in DNA repair.

In addition, unlike Wallace's findings in normal cell line TK6, we find that OGG1 overexpression promotes resistance to radiotherapy in cancer cells, as in DLD-1 cells, by increasing DNA repair. Previously in the lab, we observed that DLD-1 cells expressed higher

levels of *APE1*, *PARP1* and *POLβ* (unpublished observation). Ectopic expression of OGG1 in cells like RPE1 and TK6 cells that do not express corresponding levels of proteins involved in downstream steps of base excision repair would not be expected to confer radio-resistance and may even sensitize cells to ionizing radiation by increasing the number of DNA strand-breaks, as previously documented[119 120]. In contrast, in cancer cells that have adapted to higher level of oxidative DNA damage by increasing BER gene expression, OGG1 and CUX1 may confer protection against damage caused by ionizing radiation, as observed in DLD-1 cells (**Fig. 22**).

CUX1 Directly Affects the Recovery Rate of DNA repair

Through comet assays performed on DLD-1 and T98G cells following irradiation, we observed that CUX1 knockdown delays repair of DNA damage following irradiation (**Figs. 23 and 24**). Overall, following CUX1 knockdown cells exhibited greater damage throughout the experiment. Importantly CUX1 knockdown led to a greater amount of DNA damage even without treatment, confirming previous published observations[92], consistent with the idea that certain cancer cells might depend on CUX1 to deal with endogenously generated oxidative DNA damage. Not only do our experiments reveal that CUX1 knockdown delays DNA repair overall, they also reveal that CUX1 knockdown significantly and specifically delays repair of oxidative DNA damage (**Fig. 23 compare C to B**), shown when cells are treated with FPG prior to electrophoresis. Importantly, FPG, in addition to detecting 8-oxoG and FapyG lesions as does OGG1, also detects alkylation damage, but following ionizing radiation, FPG and OGG1 give similar number of breaks[123]. We use FPG as it is easier to work with and more effective than OGG1.

CUX1 Knockdown Sensitizes Cancer Cells to MTH1 Inhibitor TH588C while CUX1 Overexpression Confers Resistance of Cancer Cells to TH588c

To further expand on CUX1's role in oxidative DNA damage repair, we explored its potential synthetic lethality with the inhibition of MTH1, a protein involved in preventing misincorporation of oxidized nucleoside triphosphastes into DNA, hydrolyzing oxidized bases such as 8-oxo-dGTP, to monophosphates. We find that CUX1 knockdown significantly increases sensitivity to TH588c, an MTH1 inhibitor[32, 33], through clonogenic survival assays (**Fig. 25**). These results confirm that CUX1 indeed has a role in repairing 8-oxoguanine lesions surely in part via its stimulation of OGG1. In the absence of efficient MTH1 activity in preventing the

incorporation of 8-oxo-dGTP into DNA, cancer cells can still rely on elevated CUX1 levels to stimulate the removal of 8-oxoG lesions. Additionally, ectopic expression of p200 CUX1 and recombinant protein CR1CR2-NLS conferred resistance to TH588c (**Fig. 25**).

Importantly, this study demonstrates that CUX1 offers protection to cancer cells that have elevated ROS levels, typically those cells that have acquired an activating mutation in *KRAS* or *HRAS*. Activated RAS proteins allow a cascade of signals that promote erratic proliferation, prevent apoptosis, and increase metabolism, causing an accumulation of reactive oxidative species. Importantly, RAS was shown to cause the upregulation of NOX genes that code for NADPH oxidase, thereby increasing ROS levels in cells[124 125]. As can be seen in normal cells--which do not have a mutant *RAS*--CUX1 has little to no effect when it is overexpressed (**Fig. 22**). By the same token, the lack of effect of CUX1 overexpression on survival to MTH1 inhibitor TH588c of DKO-4 cell line, which forms an isogenic pair for RAS mutant gene with DLD-1 cell line, further confirm that CUX1 is only required for cells that accumulate more reactive oxidative species.

Recently two studies published by AstroZeneca have casted doubt on MTH1 as a therapeutic target, as they investigated two new compounds that inhibited MTH1 activity and yet did not affect cancer cell survival[126]. Another study reported that TH588c inhibited tubulin polymerization in addition to inhibiting MTH1, and labeled MTH1 as a nonessential protein[127]. Interestingly, CUX1 is important in the survival of cancer cells submitted to microtubule inhibitors Paclitaxel and Vincristine, as it upregulates genes involved in microtubule assembly[128]. In spite of these recent findings, and because of experimental evidence showing that CUX1 stimulates OGG1 and confers radio-resistance to cancer cells through its Cut repeats, and because Cut repeats alone confer resistance to TH588c (**Fig. 25 H and I**), I am still inclined to believe that the effect that CUX1 expression has on sensitivity of cancer cells to TH588c is due to the drug's inhibitory effect on MTH1, rather than on tubulin polymerization.

Conclusions

In conclusion, I tested and optimized various assays to measure the effect of CUX1 and OGG1 on the resistance of cancer cells to ionizing radiation.

Clonogenic assays demonstrated that ectopic expression of CUX1 or OGG1 increases survival of cells, whereas CUX1 and OGG1 knockdown decreases survival of cells in response to irradiation. Clonogenic assays also showed that CUX1 overexpression increases survival of cells, whereas CUX1 knockdown decreases survival of cells in response to treatment with the MTH1 inhibitor TH588c.

I discovered that treatment of cells with doxycycline causes a serious artifact in the methyl-^{14}C thymidine incorporation assay. Therefore I concluded that this assay cannot be used with cells that carry a doxycycline-inducible vector. However, using this assay we established that CUX1 overexpression increases proliferation of cells in response to irradiation. We also determined that OGG1 siRNA decreases proliferation of cells in response to IR.

I was able to infer that optimal viewing of samples under a wide-field epifluorescence microscope in the Single Cell Gel Electrophoresis (Comet) assay requires staining of ethanol-dried slides with SYBR green. In addition, for a more effective "No Recovery" time-point after treatment with IR, irradiation must be performed on cells that have already been embedded in agarose, in order to reduce to a minimum the repair of DNA strand-breaks. Preliminary results from comet assays indicated that CUX1 knockdown delays DNA repair of IR-induced DNA damage in T98G and DLD-1 tumor cell lines.

These experiments provide support for the proof-of-principle of CUX1 as a potential therapeutic target to sensitize cancer cells to radiotherapy.

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