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Study of DNA Double Strand Break Repair in *Dictyostelium discoideum*

Styliani Lempidaki

MSc by research in Biochemistry

Corpus Christi College

Department of Biochemistry

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The homologous recombination (HR) pathway contributes to genome integrity by mediating double strand break (DSB) repair using a homologous DNA sequence as a template. In mammals Rad51 and Brca2 are molecules central to this process. Little is known about HR repair in *Dictyostelium*. However, research previously conducted on DSB repair using this organism has shown that DSB repair pathways are highly conserved when compared to humans. This encouraged study of HR in this organism. In this study, through a bioinformatics search I have identified putative orthologues of most human HR proteins and most interestingly of BRCA2, which cannot be found in other lower eukaryotes used as models for DSB repair, such as the budding yeast *S.cerevisiae.* Brcp, the Dictyostelium BRCA2 ortholog, shows similar domain structure when compared to BRCA2-related proteins identified in other organisms. To verify the implication of HR proteins in DSB repair, I developed a method to monitor recruitment of DNA repair proteins on chromatin upon DSB induction. Findings of this study suggest that both Brcp and Rad51 get recruited to chromatin upon DSB induction and are therefore implicated in DSB repair in *Dictyostelium*. To further study Brcp function and based on findings suggesting that disruption of *brcp* might be lethal, I developed a novel system for specific and conditional depletion of endogenous *Dictyostelium* proteins. Utilizing this system, I conducted phenotypic studies in a strain depleted of Brcp to examine its role in DNA repair. Overall this study shows that the HR pathway in *Dictyostelium*

shows great similarity to vertebrates, making *Dictyostelium* an appealing model for the study of DSB repair and specifically HR.

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Abbreviations

AmpR	ampicillin resistance gene
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3 related
BER	Base excision repair
BIR	Break induced replication
BsR	Blasticidin resistance gene
C-terminal	Carboxy-terminal
CDK	Cyclin dependent kinase
DDR	DNA damage response
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-PK catalytic subunit
DSB	Double strand break
DSBR	Double strand break repair
dsDNA	double stranded DNA
G_1	Gap1
G_2	Gap2
GCR	Gross chromosomal rearrangements
HJ	Holliday Junction
HR	Homologous recombination
HRP	Horseradish peroxidase
ICL	Interstrand crosslink
IR	Ionizing radiation

Ka	*Klebsiella aerogenes*
LOH	Loss of heterozygosity
MCS	multi cloning site
MMEJ	Microhomology-mediated end joining
MR	Mismatch repair
MRN	Mre11//Rad50/Nbs1
MRX	Mre11/Rad50/Xrs2
NE	Nuclear extract
NER	Nucleotide excision repair
NHEJ	Non-homologous end-joining
NLS	Nuclear localization signal
PARP	Poly-ADP Ribose polymerase
PCR	polymerase chain reaction
PIKK	Phosphoinositol-3-kinase like kinases
PNK	Polynucleotide kinase
Rad	Radiation sensitive
ROS	Reactive oxygen species
RPA	Replication protein A
RT	Room temperature
S phase	Synthesis phase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDSA	Synthesis dependent strand annealing
SSA	Single strand annealing
SSB	Single strand break
SSBR	Single strand break repair

ssDNA	single stranded DNA
TLS	Translesion synthesis
UV	ultraviolent
XRCC	X-ray repair cross complementing
Xrs	X-ray sensitive

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Chapter 1: Introduction

1.1 The DNA Damage response

DNA is constantly being challenged by damage administered by both endogenous and exogenous agents. To ensure genome integrity and stability, cells have developed an elaborated and highly conserved signal transduction pathway, which senses damage and causes various responses based on a signaling cascade activated by DNA damage. The agents that can cause DNA damage are described below.

Endogenous damage is caused by byproducts of metabolic processes in the cell and entails base alterations and strand breakage. Examples of this type of damage is, interconversion of DNA bases caused by deamination, loss following depurination, alkylation and oxidation caused by Reactive Oxygen Species (ROS). dNTP misincorporation during DNA replication is another common phenomenon [1].

Environmental agents causing damage, can be separated in two categories. Physical agents, such as Ionizing Radiation (IR) and X-rays can cause DNA oxidation, single strand (SSBs) and double strand breaks (DSBs) whereas ultraviolent (UV) irradiation causes pyrimidine dimers and 6-4 photoproducts [1]. Chemical agents include Methyl Methanesulfonate (MMS), which causes base alkylation and subsequent SSBs as well as crosslinking agents mitomycin C, cisplatin and psoralen, used in cancer therapy, causing interstrand crosslinks. Finally, substances like topoisomerase inhibitors induce formation of SSBs and DSBs due to crosslinking of these proteins to DNA.

Cells respond to DNA damage in a strictly regulated manner that ensures repair takes place so that the cell cycle can proceed without the integrity of the genome being jeopardised. In cases where damage is extensive cells either enter senescence and stop dividing or undergo programmed cell death to avoid the harmful effects of genome instability. The DNA Damage Response (DDR) is a complex signal transduction pathway that elicits a coordinated and versatile set of responses to tackle damage (Figure 1.1). Key components of this pathway are the phosphoinositide 3-kinase related kinases (PIKKs), Ataxia Telangiectasia Mutated (ATM), Ataxia Telangiectasia and Rad3 related (ATR) and DNA-dependent protein kinase (DNA-PK) [2]. DNA-PK and ATM directly recognize DSBs, whereas ATR recognizes ssDNA coated with replication protein A (RPA). These kinases get recruited to sites of damage and through phosphorylation of downstream targets mediate cell cycle arrest, apoptosis or initiate the repair process [1, 3]. One of these targets is the C-terminus (S139) of the histone variant H2AX [4, 5]. Phosphorylated H2AX constitutes a hallmark of DSBs and is essential for recruitment of checkpoint and repair proteins to sites of damage [6]. Ultimately, ATM and ATR lead to activation of the Chk1 and Chk2 effector kinases [7]. Chk2 kinase is activated throughout the cell cycle, whereas Chk1 is only activated in response to damage during S and G2 [7]. Chk1 and Chk2 ultimately activate the tumor suppressor protein p53 [8]. Through its action, cell cycle arrest or apoptosis are induced depending on the levels of damage administered to the genome [8]. p53 exerts its checkpoint function by upregulating genes such as a ribonucleotide reductase subunit, an enzyme important for DNA synthesis during cell division which has a crucial role

in supplying precursors for DNA synthesis and whose activity is required for DNA repair in response to damage [9].

As the propagation of intact and accurate genetic information from one generation to the next is essential for organisms, cells have developed complex and interconnected mechanisms to cope with the different types of DNA damage. Each type of damage causes a different response, attracting a different set of factors to the damage site that subsequently initiate the repair process.

Mispaired DNA bases that arise during DNA replication are repaired via Mismatch Repair (MMR). The MutSα and MutSβ complexes recognize the mismatched bases and interact the Proliferating Cell Nuclear Antigen (PCNA) and its loader, the RFC complex, two complexes that are part of the replication machinery. Subsequently different MutL subcomplexes formed are responsible for repair of the mismatches [10].

Small DNA base alterations are repaired by Base Excision Repair (BER), where chemically modified bases, due to alkylation, deamination, depurination or oxidation are removed to avoid mutagenesis in subsequent DNA replication cycles. The process is initiated by DNA glycosylases and additional enzymes perform incision, gap filling and ligation. Two subpathways exist. When the short-patch pathway is employed, only one base is excised, whereas when the long-patch is utilised, at least two bases are excised [11].

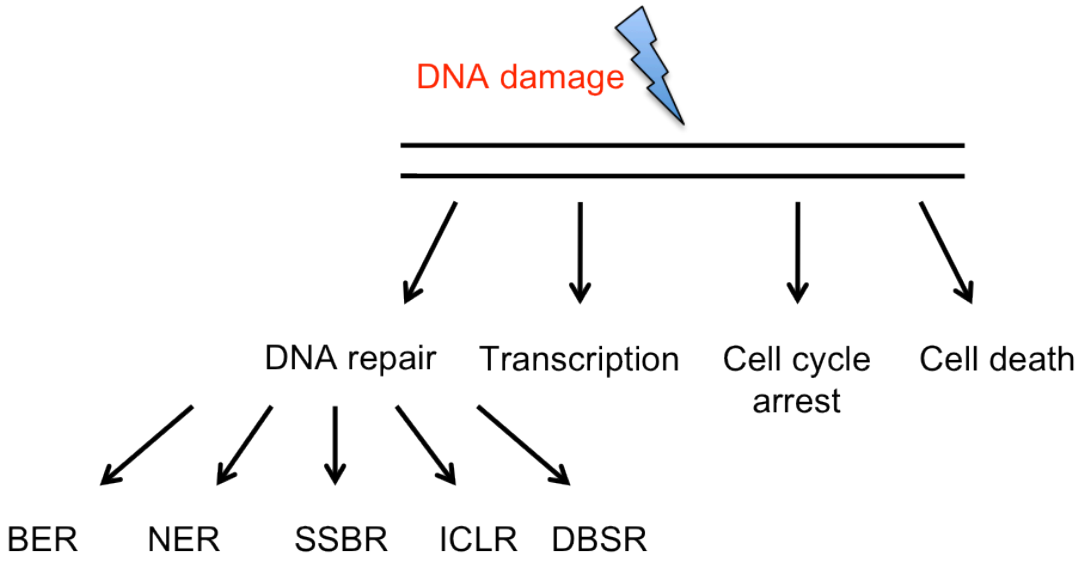


Figure 1.1: DNA damage and its consequences. When damage is administered to cells, a serious of events takes place. DNA repair is initiated and depending on the type of damage administered, different repair pathways can be employed. BER: Base excision repair, NER: Nucleotide excision repair, SSBR: Single strand break repair, ICLR: Interstrand crossling repair and DSBR: Double strand break repair. Other responses include induction of transcription of preapoptotic genes along with cell cycle arrest. When the cell cycle is arrested, cells are able to repair the damage before they go into mitosis or DNA replication, so that genetic information is inherited intact by daughter cells.

When chemical modifications distort the DNA helix structure, as happens in the case of pyrimidine dimers, caused by UV irradiation, and intrastrand crosslinks, Nucleotide Excision Repair (NER) is employed. In this pathway, a short segment of single stranded DNA (ssDNA), of approximately 30 base pairs, including the damaged bases, are removed and replaced by DNA polymerases that use the undamaged strand as a template. Transcription coupled NER (TC-NER) occurs when a distorted helix structure is encountered by the transcriptional machinery and ulitizes the Cockayne Syndrome proteins (CS-A and B), whereas all other NER is performed by the global genome NER (GG-NER) machinery through the action of the XPC-hHR23B complex [12].

Interstrand crosslinks (ICLs), which can block the progression of DNA replication by forming a physical barrier for the replisome, are repaired by the ICL repair mechanism assisted by the Fanconi Anemia associated proteins [13]. For the repair of an interstrand crosslink, the FA core complex monoubiquitinates the FANCD2-FANCI complex, which translocate to the ICL site and attracts other proteins which mediate the processing of the crosslink by nucleolytic cleavage (by SLX4, XPF and ERCC1). Bypass and repair of the damage is achieved via the action of Translesion Synthesis (TLS) Polymerases and DNA ligases [13].

When Single Strand Breaks are administered to the genome, mainly by ROS, the damaged is primarily sensed by Poly-ADP ribose polymerase 1 (PARP1). PARP1 is rapidly recruited to the damage site and modifies itself and target proteins (e.g. Histone H1) that ultimately results in attracting other proteins that

are part of the SSB repair mechanism. Damaged ends are then processed, repaired using the complementary strand of the duplex as a template and subsequently ligated by DNA ligase enzymes [14].

Finally, the most toxic type of DNA damage, is DNA Double strand breaks (DSBs). When this type of damage occurs, it can be repaired by two predominant pathways, non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ requires minimal end processing of the broken ends, which are subsequently ligated together, a process that can result in loss of genetic information. NHEJ functions throughout the cell cycle. In contrast, HR operates after extensive end processing has taken place. A homologous sequence, preferably the intact sister chromatid is used as a template for repair, resulting in error-free repair [15]. HR can only be employed during the S/G2 phases of the cell cycle, when a homologous template is available [16].

1.2 Double Strand Break Repair

DNA DSBs occur naturally during generation of antibody diversity, DNA replication and meiosis and telomere length maintenance. The central role of DSB repair in these processes makes its function essential for maintenance of genome stability and integrity [17].

DSBs can also be induced by exogenous damaging agents, such as ionising radiation (IR) [15]. Failure to repair damage can lead to cell cycle arrest and ultimately apoptosis. At the same time gross chromosomal rearrangements, such as deletions and translocations of large chromosomal segments are

observed in cells that accumulate DSBs due to aberrant DSB repair. Chromosomal rearrangements can lead to genomic instability and carcinogenesis [15].

To safeguard the genome against the deleterious effects of aberrant repair of DSBs, cells have evolved two predominant DSB repair mechanisms. Homologous Recombination (HR), which uses a homologous sequence as a template for repair and Non-Homologous End-Joining (NHEJ) which relies on minimal processing of broken DNA ends and direct ligation [15].

1.2.1 Non Homologous End Joining

NHEJ is a highly efficient repair mechanism, which operates to bring together, process and ultimately ligate the broken DNA ends. The Ku70/Ku80 heterodimer is recruited to the break site rapidly upon DSB induction. The dimer forms a ring structure, which can accommodate a DNA helix without forming covalent bonds with it [18]. Once bound, it slides inwards [19] to allow for recruitment of the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to the damage site via interaction with DNA and the C-terminus of Ku80 [2]. The structure of the DNA-PK holoenzyme has been resolved by electron microscopy, where holoenzymes bound to each of the broken DNA ends interact with each other via DNA-PKcs, tethering them together to form a synaptic complex [20, 21]. Recruitment of DNA-PKcs to the break site and interaction with DNA and Ku, induces its kinase activity, required for efficient NHEJ [22].

When a blunt end DSB is created, no processing of the break is required. However, a variety of damaging agents (e.g. IR, ROS) can cause DSBs with non-ligatable ends. 3' and 5' overhangs generated require either resection or filling in for subsequent ligation. The overhang can be used as a template DNA polymerases μ and λ as well as terminal deoxyribonucleotidyltransferase (TdT), members of the Polymerase X family, which have been shown in DNA end processing [23-25]. Although many polymerases have been implicated in filling in overhangs, only one nuclease has a role in resecting DSBs so they are rendered ligatable. Artemis was first identified for its role in end resection and exhibits a 5' to 3' exonuclease activity [26]. The importance of its function is highlighted by a study showing that lack of Artemis function results in severe immunodeficiency, due to the role of NHEJ in the creation of variable T cell receptors and antibodies (a process also known as V(D)J recombination). Also, cells deficient in Artemis are hypersensitive when treated with Ionizing Radiation (IR) [26]. Two other factors PNK and APLF also play an important role in NHEJ with both implicated in end processing [27]. WRN and its helicase activity is also implicated in NHEJ [27].

The final step for the completion of the repair process is the ligation of DNA ends by the XRCC4 /XLF/ligase 4 complex. The importance of this step is denoted by the hypersensitivity of cells to irradiation when lacking any of the two components [28]. The complex gets recruited to the break site via its interaction with the Ku heterodimer and double stranded DNA and its activity is enhanced by DNA-PKcs. A factor that seems to be important for the complex's activity, XLF, has been shown to directly interact with it to mediate non-cohesive ends ligation [29, 30].

An alternative NHEJ pathway is microhomology-mediated end-joining (MMEJ), which uses 5-25 base pairs microhomologous sequences during the aligment of broken ends, before joining. This pathway becomes active when classical NHEJ is absent and is independent of DNA-PK activity. Repair via MMEJ has been associated with chromosomal abnormalities resulting from rearrangements [31].

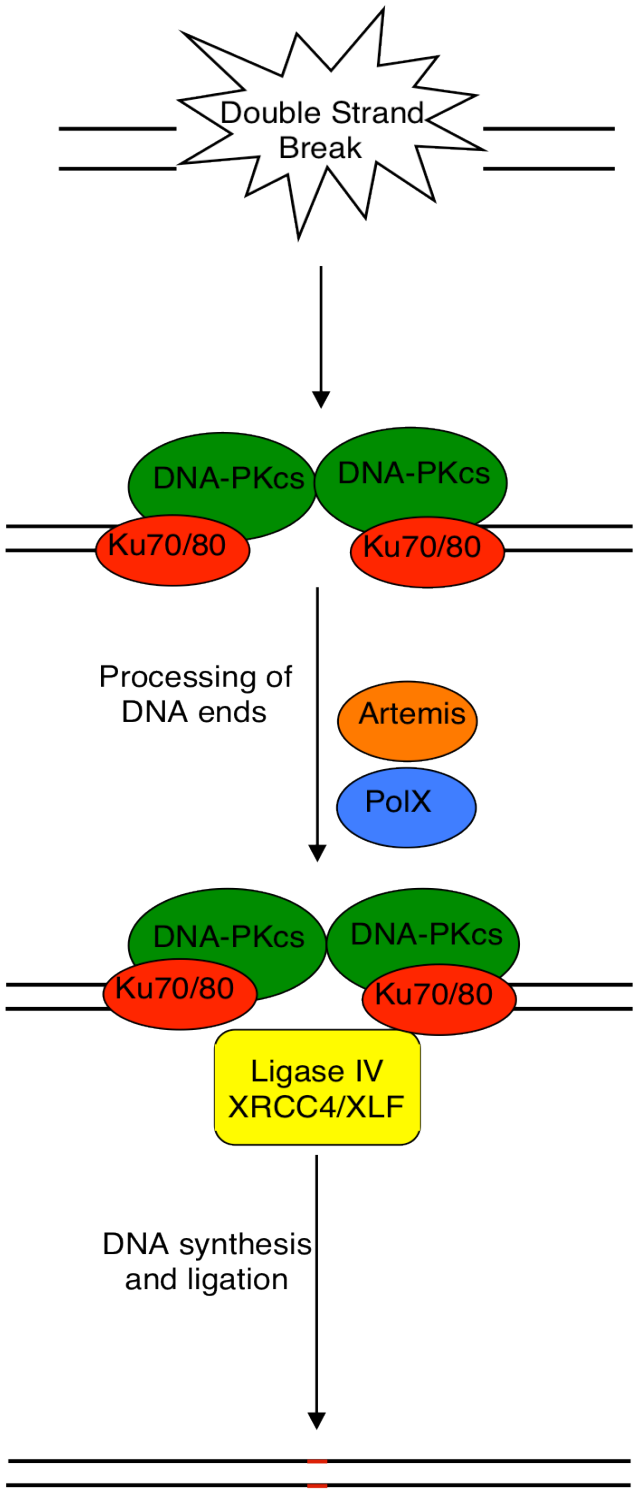


Figure 1.2: Schematic representation of Non Homologous End Joining. Upon damage induction, the broken DNA ends are initially bound by the Ku70/Ku80 heterodimer. The heterodimer then attracts DNA-PKcs. DNA-PKcs molecules interact to form a synaptic complex, to hold the DNA ends together. Upon activation of DNA-PKcs, factors are recruited to process DNA ends, namely the Artemis nuclease and/or various PolX family members. Finally, the XRCC4/ Ligase 4/ XLF complex is recruited to mediate DNA end ligation.

1.2.2 Homologous Recombination

Homologous recombination (HR) is a high fidelity, error free process, which can only take place when a homolgous DNA sequence is available to be used as a template for repair. It has a very important role both in meiotic and mitotic cells of all known organisms. In meiosis, HR functions to promote genetic diversity, as programmed DSBs are repaired by HR, a process, which results in exchange of genetic information between the paternal and maternal chromosomes in the gamete precursor. Other important roles of HR is the repair of telomeres, interstrand crosslinks and damaged replication forks [32]. In this study we focus on the role of HR in repairing DSBs induced by DNA damaging agents such as Ionizing Radiation (IR) and radiomimetic drugs (e.g. phleomycin).

The time window during which HR is active is tightly regulated by the cell cycle machinery. In mitotic human cells, HR is downregulated during G_0 and G_1 phase of the cell cycle, where NHEJ is the predominant pathway for repair [15]. When HR is upregulated during the S and G_2 phases, NHEJ continues to be active and evidence of direct competition between the two pathways exists. More specifically, it has been shown that Ku70/Ku80 heterodimer binds to DNA ends during the S phase to prevent their nucleolitic processing, thus promoting NHEJ over HR [33].

The Double Strand Break Repair model

According to the Double Strand Break Repair (DSBR) model for recombination, which was proposed many years ago [34], there are three stages in the HR

repair process: presynapsis, synapsis and postsynapsis. Although the model has been modified since its conception, the key steps of recombinational repair remain the same (Figure 1.3). DSB induction initiates the process of HR and resection of DNA ends takes place. When DSBs occurring in the vicinity of repetitive sequences are being repaired, the ends are resected and directly annealed to each other. Repair is completed with resection of the flaps resulting from the direct ligation of the two ssDNA molecules. This pathway is termed Single Strand annealing and is an error prone process that results in deletion of repeats [35].

In DSB repair of mitotic cells, extensive end processing results in formation of 3' ssDNA tails, which are subsequently coated with a recombinase protein [36]. This structure then performs a search for a homologous sequence, which it subsequently invades to form a three stranded structure, the Displacement loop (D-loop) [36]. The 3' end of the invading strand then primes DNA synthesis by DNA polymerases using the intact complementary strand of the heteroduplex as a template [36]. Depending on the pathway employed downstream of D-loop formation and extension of the invading strand, different sets of proteins are required for completion of HR [36].

In the case of synthesis-dependent strand annealing (SDSA) after extension of the 3' end of the invading strand the former is displaced from the duplex DNA and anneals to the second end of the DNA break [36]. The newly synthesized strand is used as a template for the repair of the complementary strand [36]. This repair pathway results in repair that does not involve exchange of genetic material between the two homologous chromosomes.

In the case of single ended DSBs, Break induced replication takes place. More specifically, only one end of the DSB invades the homologous duplex and upon extension of the 3' end a replication fork is formed within the displacement loop. BIR can be particularly harmful for a cell since this mode of repair can result in loss of heterozygosity, a phenomenon observed when the homologous chromosome's sequence is used as a template for repair of both strands of the broken chromosome. Thus leading to loss of the allelic information contained in one of the chromosomes [37].

Finally, when the second end of the DSB is captured by the displacement loop, an four-stranded intermediate structure called a double Holliday Junction (dHJ) is formed (Figure 1.3), where the two DNA molecules remain covalently linked until the dHJ is resolved by one of two mechanisms: Via the action of Bloom syndrome (BLM) helicase in complex with Topoisomerase IIIα (TOPOIIIα), dissolution of Holliday junctions is achieved, a process that always results in no exchange of genetic information between the two sister chromatids (non-crossover). On the other hand, HJ resolution mediated by the nucleases MUS81-EME1, SLX1 and its scaffold SLX4 and GEN1 is a process that can result in both exchange of genetic information between the two homologous sequences (crossover) or no exchange (non-crossover). The outcome of this process depends on the manner the HJ is cleaved by the aforementioned nucleases [38]. All the above are summarized in Figure 1.3.

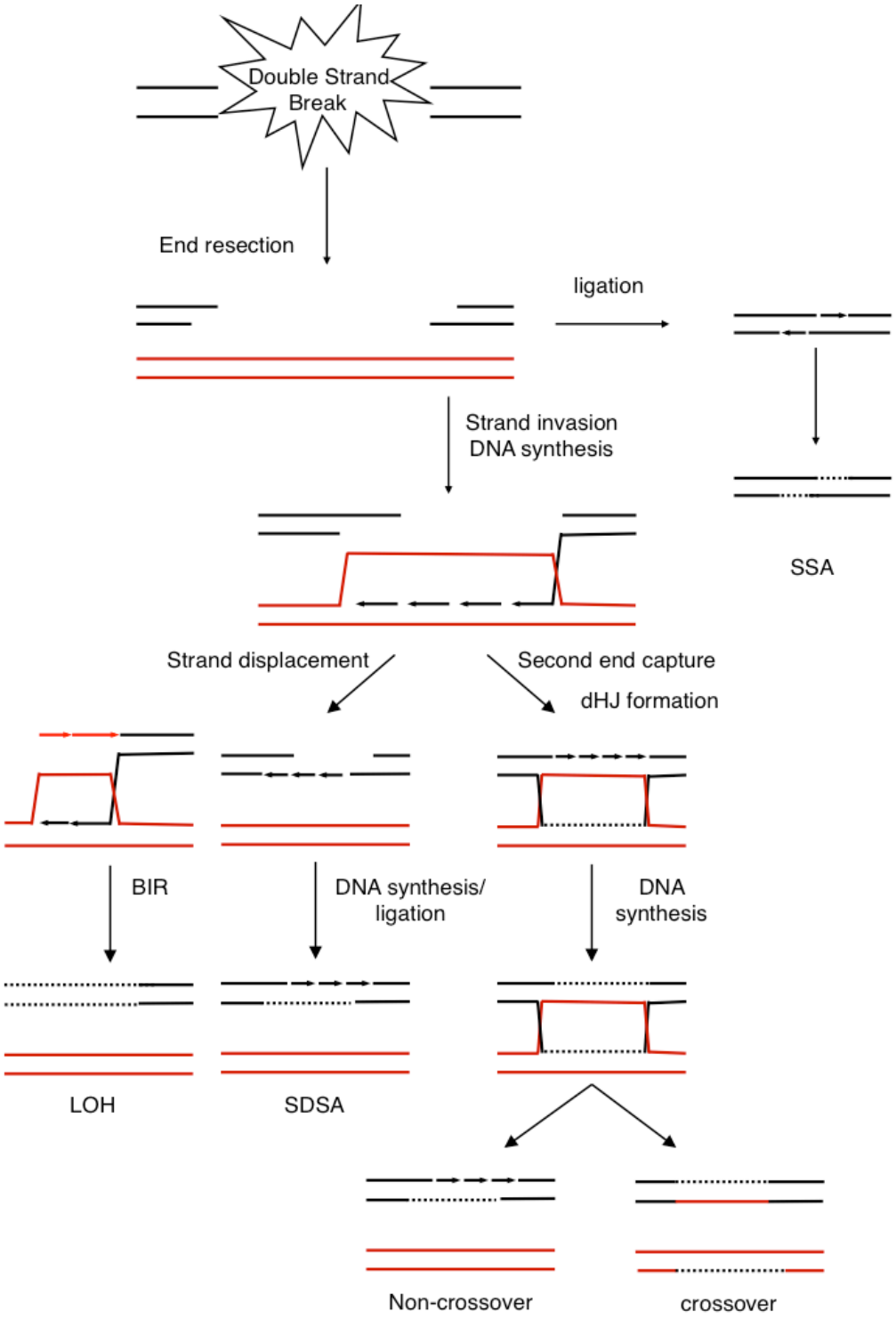

Double Strand Break
End resection
ligation
Strand invasion
DNA synthesis
SSA
Strand displacement
Second end capture
dHJ formation
BIR
DNA synthesis/
ligation
DNA
synthesis
LOH
SDSA
Non-crossover
crossover

Figure 1.3: A schematic representation of the different possible outcomes of homologous recombination mediated repair of DNA double strand breaks. After a double strand break is induced, end resection follows, leading to the formation of 3' overhangs. When the ends contain direct repeats, the overhangs anneal to each other and can be ligated by single strand annealing (SSA), leading to loss of genetic information. In all other cases, one of the 3' ends invades a homologous sequence, forming a Displacement loop (D-loop) and primes DNA synthesis, which uses the homologous sequence as a template. In the case of single ended DSBs, break induced replication (BIR) takes place, leading to loss of heterozygosity (LOH). When the newly synthesized sequenced is displaced from the D-loop without the second end of the DSB being captured by it, synthesis-dependent strand annealing (SDSA) takes place where the newly synthesized strand is used as a template for repair of the complementary strand. When the second end of the DSB is captured by the D-loop, a structured termed a double holliday junction (dHJ) is formed and its resolution can either lead to exchange of genetic information between the two chromosomes (crossover) or not (non-crossover).

1.2.2.1 Presynapsis

DSB repair pathway choice and initial resection by the MRN-CtIP-BRCA1 complex

In the presynaptic phase, extensive processing of the broken DNA ends takes place. A key player in initiation of end resection is the MRN complex, which is recruited to DSBs only seconds after their formation [39]. Mre11 and its 3' to 5' exonuclease activity is essential for end resection [40]. However, Mre11 does not act alone. CtIP, a protein conserved from lower eukaryotes to humans (Ctp1 in *S.pombe,* Sae2 in *S.cerevisiae*), has recently been identified as an essential component for the initial resection of DSBs [41, 42]. Findings in yeast suggest that end resection is a stepwise process where initial resection is mediated by the MRX complex and Sae2 and followed by extensive resection by the Exo1 nuclease and the Sgs1 helicase [43]. In the absence of Sae2 HR is impaired, indicating its important role in the initial steps of HR [43]. A key study by *Limbo et al.* [42] in *S.pombe* shows that Ctp1, a distant ortholog of Sae2 and an ortholog of CtIP, is cell cycle regulated and contributes in end resection, which is only promoted during the S/G_2 phase of the cell cycle [42]. The same study shows that the MRN complex and Ctp1 act in the same pathway, since mutation of the latter has an epistatic relationship with mutation of MRN components [42]. The same is true for the human counterparts MRN and CtIP [44]. Human CtIP, like its yeast counterpart (Sae2 and Ctp1) acts to resect DNA at the break site, during the initial steps of HR, in concert with the MRN complex [41] (Figure 1.4).

In G_2 and S phase, where two copies of each chromosome exist, CtIP is activated via CDK1-mediated phosphorylation, which promotes end resection

and ultimately homologous recombination [42, 44]. Substitution of the amino acid targeted by CDK1 for phosphorylation with one mimicking phosphorylation renders CtIP constitutively active and resection is carried out even in phases of the cell cycle other than G_2/S [44]. The above displays how CtIP phosphorylation by CDK1 during the S phase is a key switch from NHEJ to HR. Once DSBs undergo nucleolitic processing and 3' ssDNA tails are generated, repair is channeled towards HR. Because homologous DNA sequences are not readily available throughout the cell cycle, pathway choice must be strictly regulated depending on the cell cycle stage. When cells are in G_1 phase, where a homologous template is absent, NHEJ is the preferable repair pathway and end resection is minimized [45].

An interaction that is important for the aforementioned CtIP activity that is dependent on its phosphorylation by ATM at S327, is with BRCA1 [46]. BRCA1 also associates with the MRN complex to promote end resection [47]. From the above it can be concluded that the MRN-BRCA1-CtIP complex mediates the initial steps of DNA end resection of DSBs administered during the G_2/S phase.

Extensive resection by Exo1 and DNA2 assisted by BLM

Although MRN-CtIP are important for initial end resection, a second phase of end resection is required so that long 3' ssDNA tails are generated. Research in *S.cerevisiae* has identified Exo1 and Dna2 nucleases along with Sgs1 helicase as important for this step [43]. In humans, two different mechanisms for resection have been identified, both of which involve the BLM (Bloom Syndrome protein) helicase one of the human orthologs of Sgs1. BLM with its helicase activity unwinds DNA, which then becomes accessible by EXO1 and

DNA2 for nucleolitic processing [48]. Both of these complexes are needed for the extensive end resection. Once the long ssDNA strands are generated, they are coated by RPA and stabilized [49]. ssDNA-RPA is a substrate for the Rad51 recombinase, which will subsequently displace RPA and bind to ssDNA to form a nucleoprotein filament (Figure 1.4).

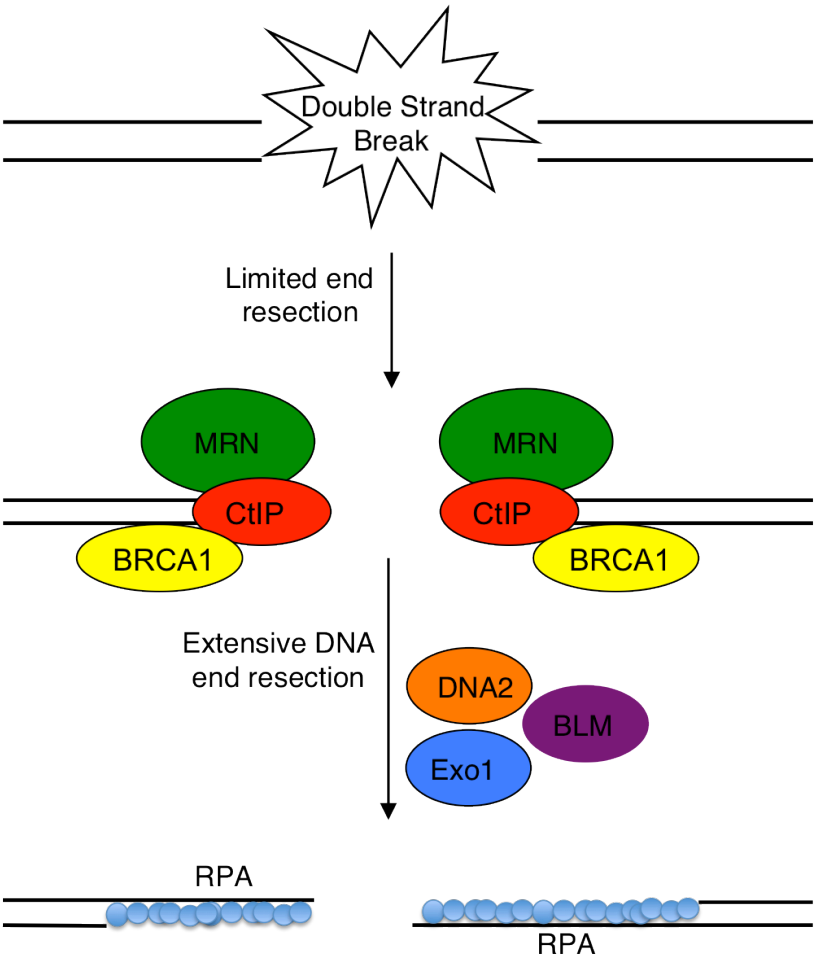


Figure 1.4: A schematic representation of the end resection process during the repair of a DNA double strand break via homologous recombination. When a DSB is administered to the genome, it is recognized by the MRN complex. During the G_2/S phase of the cell cycle CtIP is also recruited to the site of damage, activated by CDK1. MRN and CtIP perform limited end resection which is required for subsequent processing of the break by the HR machinery. An interaction important for efficient initial resection is that of BRCA1 and CtIP. This interaction is dependent on phosphorylation of CtIP by ATM. When initial resection is completed, extensive resection mediated by Exo1, DNA2 and BLM takes place. This step results in the generation of extensive threads of ssDNA covered with RPA, an important intermediate for initiation of HR and a substrate for Rad51 recombinase.

The Rad51 recombinase and its mediators and the formation of the nucleoprotein filament.

Rad51 is a dsDNA-dependent ATPase, which has been shown to function in DNA repair [50, 51]. It's bacterial counterpart, RecA has been studied extensively and shown to form a nucleoprotein filament with ssDNA and to participate in homology search and strand invasion [52]. Rad51 was first identified in yeast as a RecA-like protein [50]. Its human counterpart was later identified as a protein that promotes strand transfer *in vitro* [53]. *In vivo*, cells lacking Rad51 exhibit spontaneous and severe genome instability and eventually die [54]. Rad51 forms distinct nuclear foci upon damage induction in human cells, which are believed to represent sites of active HR [55].

At the molecular level, Rad51 functions to form a helical nucleoprotein filament, by associating with ssDNA while displacing RPA, which functions to stabilize the ssDNA by removing any secondary structures. Through live cell imaging, the dymanics of the nucleoprotein filament formation have been determined and it was shown that when Rad51 is bound to DNA, the helix is extended over 60% compared to free DNA [56]. This nucleoprotein filament has the ability to invade homologous sequences with a 3' to 5' polarity and search for homology, ultimately forming a stable heteroduplex by annealing with the one strand and displacing the complimentary strand [57].

Rad51 assembles on ssDNA and forms a nucleoprotein filament. The displacement of RPA from ssDNA as well as the formation of the nucleoprotein filament, are incredibly inefficient when only Rad51 is present [51]. For rapid and efficient nucleoprotein filament formation the action of a mediator proteins

is required. Different classes of Rad51 mediators have been described, but the relationship between them is not clear. Firstly, the five known Rad51 paralogues (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3) have been shown to have significant sequence similarity with Rad51 but cannot perform strand invasion independently of Rad51 [51]. Their role is accessory to Rad51 and they exist in two distinct complexes that stabilize the nucleoprotein filament prior to strand invasion [58].

Another mediator of Rad51 is the Rad52 protein, which has been shown to promote Rad51 activity *in vitro* [59]. Although yeast Rad52 is essential for efficient HR and *rad52* mutants are extremely radiosensitive, Rad52 deficient vertebrate cells show little to no phenotype [60]. This observation denotes how the Rad51 mediator role is played by different proteins in vertebrate cells. Indeed, BRCA2 another type of Rad51 mediator plays the most important role in its recruitment to ssDNA via displacement of RPA along with its interacting protein PALB2 [61, 62] (Figure 1.5). The role of BRCA2 in DNA repair is analyzed in detail in section 1.3.3.

1.2.2.2 Synapsis

Once Rad51 has formed a nucleoprotein filament with the ssDNA, a homology search is performed, where the filament captures a duplex DNA molecule and scans it for regions of homology. Rad54, which belongs to the Swi2/Snf2 superfamily of proteins, is a dsDNA-dependent ATPase translocase and chromatin remodeling factor that plays an important role in this process [63]. It has been shown to directly interact with Rad51 and enhances its ATPase activity [63]. Rad51 along with RAD54 promotes the formation of a

displacement loop at the target homologous sequence, forming heteroduplex DNA [63] (Figure 1.5). RAD54B a paralog of RAD54 showing sequence and function homology, has also been shown to be an accessory factor in this process [64]. Notably, mutation of either RAD54 or RAD54B confers mild or no sensitivity to DNA damaging agents. However, double mutants are extremely sensitive [64]. The five Rad51 paralogues are believed to stabilize the heteroduplex and mediate its formation, based on biochemical studies [58].

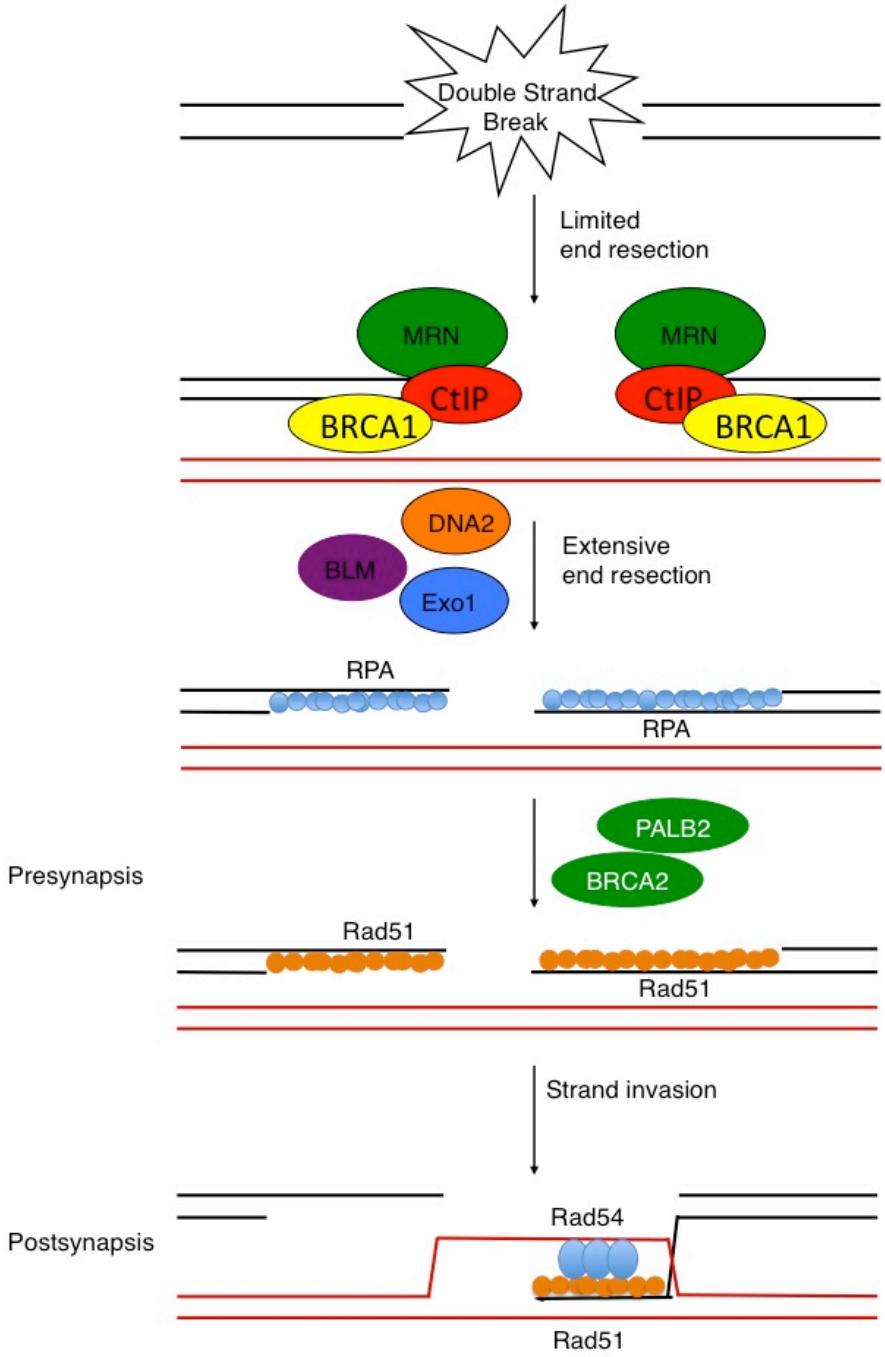


Figure 1.5: Schematic representation of the initial stages of HR. Upon initial and extensive end resection (described in 1.4), BRCA2 and PALB2 mediate recruitment of Rad51 on ssDNA by displacing RPA. Subsequently, Rad51, assisted by Rad54 performs a homology search and invades the homologous sequence to prime DNA synthesis for the repair process to be initiated.

1.2.2.3 Postsynapsis

Once the heteroduplex is formed, the 3'-OH end of the invading strand is used to prime DNA synthesis using the strand it is annealed to as a template. Resolution of the D-loop follows and can lead to different outcomes depending on the HR pathway employed. In some cases, the second 3' end of the DSB is captured by the D-loop and used as a primer for DNA synthesis by the strand of the sister chromatid displaced during D-loop formation. This results in formation of a double Holliday Junction, where both sister chromatids are connected. Its resolution can lead to reciprocal exchange of genetic information flanking the initiation site. In order for the structure to be resolved, the action of various proteins has been shown to be required. More specifically, Mus81/EME1 as well as GEN1 and SLX4 have been shown to play a role in dHJ resolution [65]. Additionally, BLM helicase has been shown to have a role in resolving dHJ during repair of damaged replication forks [66]. However, the generation of crossover is a rare event in somatic cells [67]. Alternatively, breaks are repaired via BIR or SDSA. The three subpathways are described in more detail in section 1.3.2.

1.2.3 BRCA2

1.2.3.1 The cellular function of BRCA2

The breast cancer susceptibility 2 (BRCA2) gene was first identified as a tumor suppressor associated with early-onset familial breast cancer [68]. Although its cellular function was unknown, it was clear that individuals carrying germline mutations that disrupted the function of the encoded protein were predisposed

to developing breast or ovarian cancer at some stage during their lifetime with a probability exceeding 70% [69].

Early studies that implicated BRCA2 in DNA repair demonstrated how depletion of BRCA2 causes early embryonic lethality associated with defective DNA repair and genome instability in mice [70-72]. In mouse cells, upon BRCA2 inactivation, gross chromosomal rearrangements can be observed [73]. Moreover, the expression of a truncated form causes chromosomal abnormalities and reduced replicative index of the mutant cells [74]. More detailed analysis showed that BRCA2 exerts its function through interaction with Rad51 [70, 71]. Supporting this finding, when cancerous human cells with a hypomorphic form of BRCA2 are complimented with the wild type protein, HR is upregulated in a Rad51-dependent manner, while other repair pathways remain unaffected [75]. From the above, it became apparent that BRCA2 is involved in HR-mediated repair. A clear indication that BRCA2 functions to facilitate Rad51-mediated HR came when *Tarsounas et al.* [76] observed that Rad51 foci formation, upon DSB induction, was diminished when BRCA2 was absent in human cells.

Biochemical, bioinformatic and structural analysis of BRCA2 has shed light on BRCA2 function in mammalian cells. The human BRCA2 protein is 3,418aa long and its sequence does not resemble that of any other known human protein. Human BRCA2 contains eight internal repeats termed BRC repeats, all of which are encoded by exon 11 [77]. The repeats are also conserved in other mammals, allowing for study of the cellular function of the protein in other mammals [78]. These repeats have been shown to mediate interaction with

Rad51, regulating its localization an association with ssDNA [79, 80]. Experiments where BRC4 was expressed in BRCA2 deficient cancer cells show a dominant negative effect of the over expression on BRCA2-RAD51 interaction, which lead to radiosensitivity and impaired Rad51 damaged-induced foci formation [81]. These findings indicate that BRC repeats interact with Rad51 to mediate its function in HR. The C-terminus of the protein has also been shown to interact with Rad51 [70, 71, 82]. It is interesting how the C-terminal Rad51-interacting domain of BRCA2 only interacts with the nucleoprotein filament and not with the monomeric protein, indicating a role for it in filament stabilization, rather than recruitment of Rad51 to ssDNA-RPA filaments [83]. This interaction is abrogated in a CDK dependent-manner, when kinases phosphorylate S3291 of the C-terminus of BRCA2 [84]. During S phase, the levels of phosphorylation are low. As cells progress into mitosis, BRCA2 becomes increasingly phosphorylated and unable to interact with Rad51, a mechanism that offers additional control over the HR pathway. Another important feature of the C-terminal portion of the BRCA2 is the existence of three Oligonucleotide-Oligosaccharide Binding folds. These folds are similar to those present in RPA when superimposed to the 3D structure and retain the conserved function of binding ssDNA with high affinity [85].

Research conducted with BRCA2-related proteins in other organisms has shown that BRCA2 function is highly conserved [74, 86-90]. However, the size of BRCA2-related proteins varies greatly, with the human protein being the biggest, 3418aa long, while the *C.elegans* ortholog is the smallest, of only 383aa [61, 88]. Nonetheless, the BRC repeats are highly conserved in most eukaryotes [78, 86-90] and are approximately 30aa long. In *Dictyostelium* the

repeats are only 20aa long (this study). However, even in the smallest of repeats, the amino acids deemed to be important for interaction with Rad51 by structural studies are conserved [91].

Isolation of full length human BRCA2 has proven to be challenging, due to its large size. However, recently *Jensen et al*. [61] purified the full length protein and perform various biochemical assays which provided invaluable information on the function of the protein as a mediator of Rad51. Specifically, BRCA2 functions to bind Rad51 and potentiates recombinational DNA repair by promoting assembly of Rad51 on ssDNA. BRCA2 targets Rad51 to ssDNA preventing its binding to dsDNA and enables it to displace RPA from ssDNA. Finally, it stabilizes the ssDNA-Rad51 filaments by preventing the hydrolysis of ATP bound to Rad51 (Figure 1.6) [61].

1.2.3.2 BRCA2 and cancer

Germline mutation of BRCA2 is mostly associated with hereditary breast and ovarian cancer [69, 92]. Accordingly, people carrying germline mutations of the protein displaying a high probability of developing these types of cancer [92]. The risk for developing one type of cancer against the other tends to vary depending on the position of the mutation within the BRCA2 molecule. It is indicative how mutations at the Ovarian Cancer Cluster Region of BRCA2 (OCCR: nucleotides 4,075-6,503) predispose patients to ovarian cancer, while the risk for breast cancer is low [93]. Most characterized mutations involve small insertions or deletions that alter the reading frame, introducing frame-shifts and in many cases, premature stop codons that result in the expression of truncated

BRCA2 lacking the C-terminus, where the NLS is located [92]. Deletion of the C-terminus results in inability of the mutant protein to translocate to the nucleus, where BRCA2 normally functions. Other characterized mutations introduce premature stop codons that truncate the protein at various positions within exon 11, in which the BRC repeats are encoded [92]. In this case, loss of BRC repeats along with the C-terminal domain cause expression of a hypomorphic form of BRCA2 that cannot exert its function in HR [92]. Finally, the 999 del5 mutation, a common BRCA2 mutation found in the Icelandic population, causes truncation of the protein at nucleotide 999 located in exon 9, rendering the protein non-functional [92]. All of the above mutations, due to their impact on BRCA2 function, cause genomic instability that subsequently leads to carcinogenesis.

It is interesting how cells derived from Fanconi Anemia patients, harbor biallelic inactivation of BRCA2 expressing truncated or mutated forms of the protein [94]. However, breast, ovarian and prostate cancer risk is lower than patients carrying heterozygous germline mutations [94].

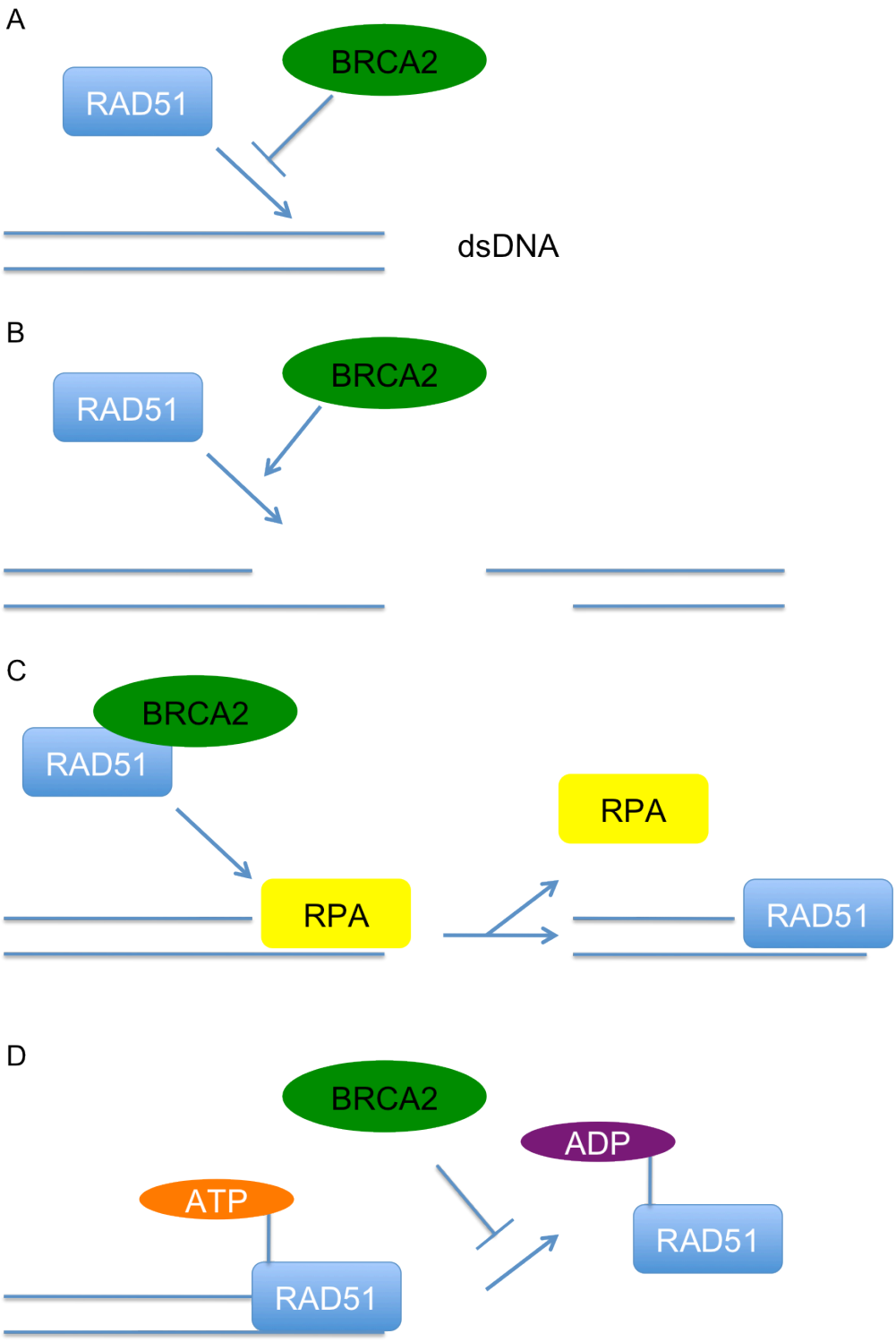


Figure 1.6: The molecular functions of BRCA2. BRCA2 mediates the formation of RAD51–single-stranded DNA (ssDNA) in four ways. **(A)** It inhibits the binding of RAD51 to double-stranded DNA. **(B)** It stimulates the binding of RAD51 to single-stranded DNA tails at DNA double-strand breaks. **(C)** It helps RAD51 to bind to ssDNA in the presence of the RPA protein. **(D)** It inhibits RAD51 dissociation from ssDNA mediated by ATP hydrolysis to ADP.

To further analyze BRCA2 function *Carreira et al.* [95] proceeded to analyze the specificity of each of the BRC repeats for binding Rad51. Their finding show two classes of repeats. The first 4 BRC repeats (1,2,3 and 4) have high affinity for free Rad51 and when bound to it, they reduce its ATPase activity. Repeats 5,6,7 and 8 have high affinity for Rad51 bound to ssDNA, to which they bind without altering its activity. The authors conclude that combination of the two different activity results in efficient control of the formation of the nucleoprotein filament.

Recently a partner and localizer of BRCA2 (PALB2) has been identified [62]. PALB2 is a protein that binds BRCA2 and increases its stability while promoting its translocation to the nucleus, where it can exert its function in DSBR. BRCA2 and PALB2 form distinct damaged-induced foci that colocalize. When PALB2 is absent or when the PALB2 interacting domain of BRCA2 is mutated, BRCA2 levels drop, due to targeting of the unstable protein to the proteasome for degradation. At the same time, BRCA2 is unable to translocate to the nucleus and form damage induced foci. Interestingly, PALB2 depletion phenocopies BRCA2 deficiency [62]. All the above denote an important function of PALB2 for BRCA2 stability [62]. Importantly, PALB2 and BRCA2 are part of a complex also containing BRCA1, which appears to be an upstream regulator of BRCA2, as its depletion abrogates foci formation of the two proteins [96].

Another protein that interacts with BRCA2 and PALB2 is the breast cancer susceptibility 1 (BRCA1) protein. In late stages of HR it functions to localize BRCA2 and PALB2 to sites of DNA damage, where focal accumulation of the three proteins can be observed [96]. Apart from its role in HR repair, BRCA1

has been shown to participate in other fundamental cellular processes namely checkpoint activation, DNA replication and end resection during the primary stages of HR (described in section 1.3.2.). For all these functions BRCA1 forms distinct complexes [97].

1.2.3.3 The synthetic lethality model

As explained in section 1.2.3.1, BRCA2 deficient cells cannot perform HR. This deficiency has recently been exploited to specifically target BRCA2 deficient cancer cells, by targeting another repair pathway and relying on the synthetically lethal relationship resulting from inactivation of both pathways. More specifically, Poly (ADP-ribose) polymerase 1 (PARP1) is a sensor of SSBs [14]. Upon SSB induction, PARP1 is rapidly recruited to sites of damage and transfers ADP-ribose chains to various substrates in its vicinity, including itself [14]. The activity of PARP1 then signals the damage to downstream factors that facilitate repair [14]. When PARP1 is inhibited, sensing of SSBs is defective, resulting in impairment of the repair process [98]. When cells enter the S phase of the cell cycle with unrepaired SSBs and undergo DNA replication, these breaks are transformed to DSBs [98].

In wild type cells, repair of these breaks is mediated by HR. However, in cells where BRCA2 has been deactivated, DSBs will remain unrepaired or will undergo illegitimate repair, leading to accumulation of damage and chromosomal instability (Figure 1.7) [73, 75]. This synthetic lethal relationship has been exploited for the development of PARP inhibitors as potent anti-cancer drugs against BRCA negative cancer cells [98].

PARP inhibitors are a very promising drug for treatment of cancers based on HR deficient cells. At the moment, clinical trials are conducted in which PARP inhibitors are being used in combination with other drugs as a treatment for many types of cancers, since genomic instability and defects in DNA repair is a hallmark of cancer in general [99].

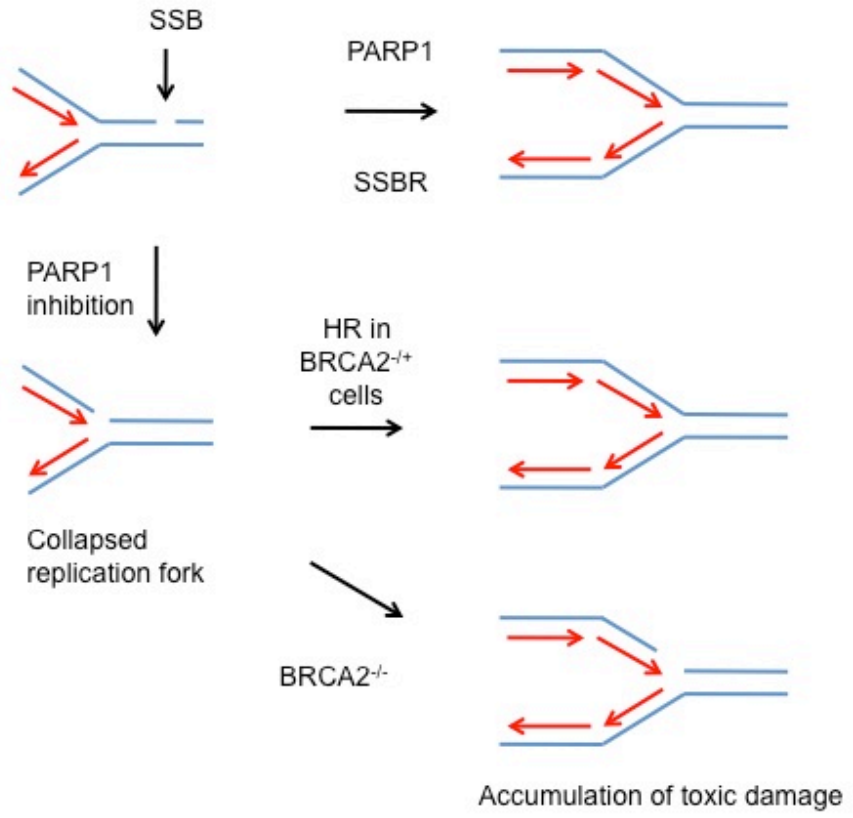


Figure 1.7: A schematic representation of the synthetic lethality model. When SSBs are induced they are sensed by PARP1 and repaired via SSBR. However, when PARP1 is inhibited by the use of PARP inhibitors, damage of this type persists and turns into DSBs, when cells enter the S phase of the cell cycle. In BRCA2 proficient cells, this type of damage is repaired by HR. When BRCA2 is mutated, HR is deficient. As a result, DSBs accumulate and cancer cells accumulate toxic damage which eventually lead to cell death.

1.2.4 *Dictyostelium* as a model for DSB repair

The social amoeba *Dictyostelium discoideum* is a soil dwelling, haploid, unicellular organism, that feeds on bacteria. When a food source is available, cells divide mitotically, undergoing the vegetative cycle. Upon starvation, *Dictyostelium* enters a developmental stage, where a multicellular aggregate is formed after secretion of cyclic AMP from cells. The cAMP gradient formed acts as a chemoattractant. Through a defined developmental process which involves changes in gene expression, cells adopt two different cell fates and ultimately form a fruiting body containing a spore head, supported by a stalk (Figure 1.8) [100]. When the conditions become favorable again, spores hatch to return to the amoeboid stage [101].

Through random mutagenesis of the original wild-type strain, NC-4, the Ax2 and Ax4 strains, that can grow axenically in a defined medium, have been generated and are used in laboratories [102]. These strains can be genetically manipulated with remarkable ease, a feature that has made *Dictyostelium* an attractive model organism since it is also unicellular, haploid and can be grown relatively easily in the laboratory.

Recently the genome of the organism has been fully sequenced [103] and a genome database created (www.dictybase.org) [104]. The *Dictyostelium* genome comprises of 12,500 genes and 34Mb of genome, spread over 6 chromosomes. It is remarkable that 75% of all bases pairs are A-T, revealing an AT bias for the genome.

Sequencing also allowed for evolution studies to be performed. According to these, *Dictyostelium* has branched off the line leading to metazoans after plants but before fungi. However, it appears that *Dictyostelium* has diverged less than fungi retaining proteins and conserved domains that are present in humans but not fungi [103]. An example of a signaling protein cascade that exists in *Dictyostelium* but is absent in fungi is phosphotyrosine signaling. This process was previously thought to exist only in metazoans. Its conservation in *Dictyostelium* suggests a longer evolutional history for this particular pathway [103]. Although *Dictyostelium* is not a very common model, the resemblance of some cellular processes to those of vertebrates has made it an invaluable tool to study these processes. More specifically, *Dictyostelium* has been used for many years as a model organism for the study of chemotaxis, cell differentiation and other basic cellular processes yielding results that have assisted in understanding of cell to cell signaling and altered gene expression in response to environmental factors [100, 105].

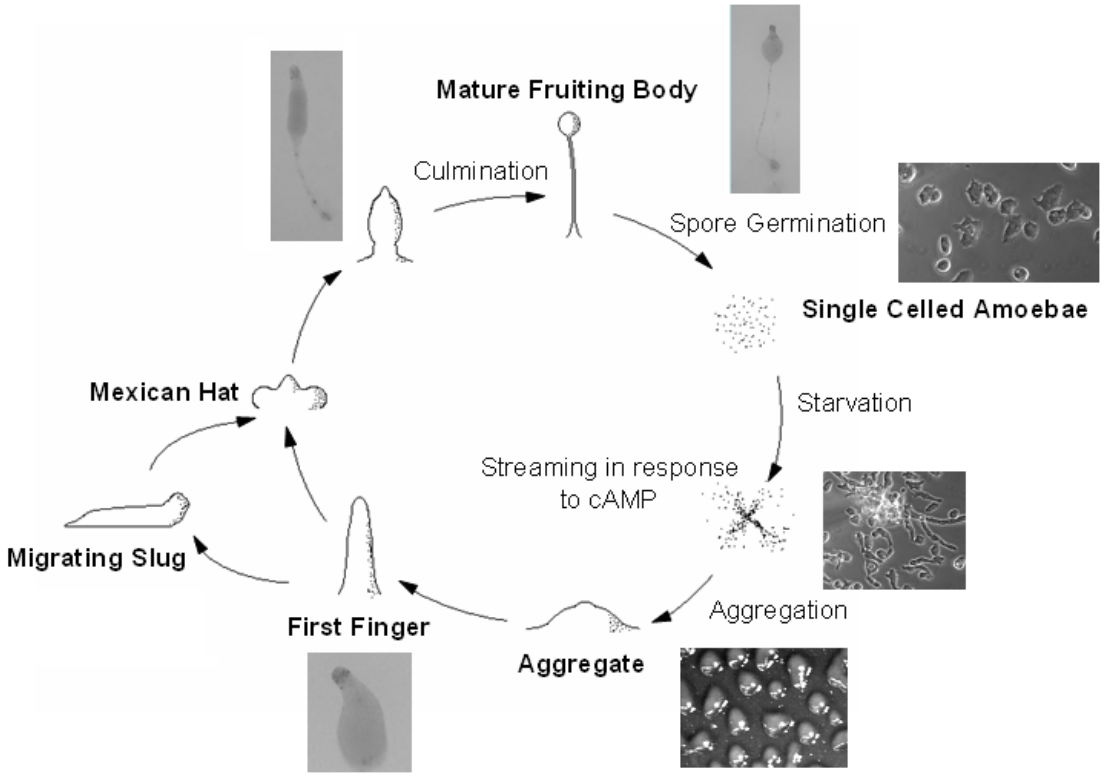


Figure 1.8: The *Dictyostelium* life cycle. The organism spends most of its lifetime in the vegetative cycle. When starved, cells undergo either the social or sexual cycle. When under the social cycle, secretion of cyclic AMP, a chemoattractant, causes aggregation of cells. The aggregate passes many morphologically distinct phases, which ultimately lead to the differentiation of cells to either stalk or spore cells through defined changes in gene expression. Cell fate decision is governed by various factors including nutritional history and cell cycle stage. At the slug stage, prestalk and prespore cells have already formed and migrate to the anterior and posterior of the slug respectively. Culmination leads to the formation of a mature fruiting body. Cells forming the stalk are sacrificed to support the spore cells, which are enclosed in a hask. When conditions become favorable again, spores germinate and the amoebae return to the vegetative cycle (Figure is courtesy of Dr. Cathrine Pears [106]) .

Regarding DNA repair in *Dictyostelium* early findings by *Deering et al.* showed that the organism is extremely radioresistant [107]. In subsequent experiments radiosensitive mutants were identified [108]. However, the identification and study of distinct repair pathways only became possible later. Through *in silico* studies proteins implicated in DSB repair pathways were identified and subsequently validated. More specifically, orthologs of mammalian DNA-PK and the nuclease Artemis have been identified and studied in this organism [109, 110]. This finding is particularly striking, as these components were, until recently, thought to be conserved only in vertebrates. At the same time, putative orthologs of all NHEJ core components [111] have been identified and the utilization of NHEJ throughout the cell cycle analyzed [109]. Another important finding regarding NHEJ is that the ortholog of PARP, Adprt1a is required for efficient NHEJ, a function that is mediated by its interaction with the PAR Binding Zinc-Finger (PBZ) domain at the C-terminal portion of Ku70. Interaction of PAR chains and Ku70 is important for efficient NHEJ, as shown by the increased sensitivity of *adprt1a* mutant strains when treated with DSB inducing agents [112]. Another interesting observation is that *Dictyostelium* possesses a minimal FA pathway [113].

Experiments conducted have assisted in determining the relative contribution of HR and NHEJ in DSB repair in *Dictyostelium* vegetative cells. Interestingly, these cells require an intact HR mechanism, as mutation of Exonuclease 1 sensitizes the cells after DSB administration [110]. This can be largely explained by a recent finding confirming earlier data showing that vegetative cells are mostly in the G2 phase of the cell cycle [114], where a sister chromatid is available and error free HR is possible [16].

All the above make *Dictyostelium* an appealing model for study of DSB repair. However, many more aspects of DSB repair need to be explored for us to gain a better understanding of the organism's defense mechanisms against DNA damage.

1.2.5 Aims of this study

As is obvious from the above section, study of NHEJ in *Dictyostelium* has been a lot more extensive compared to HR. HR plays a fundamental role in repair of DSBs but also the general biology of the cell [115, 116]. Although *Dictyostelium* are haploid, HR still remains an important pathway for repair of DSB, stalled replication forks and interstrand cross links, making HR integrity an imperative for genome stability and cell survival.

In vertebrates, BRCA2 is identified as a tumor suppressor gene, playing a pivotal role in HR by acting as a mediator of Rad51 and facilitating the most central event to the repair process, the strand invasion step [117]. Cells mutated in BRCA2 are unable to perform HR, develop genome instability and ultimately become cancerous [73, 75].

Recently, the synthetically lethal relationship of PARP1 and BRCA2 was exploited in experiments where specific killing of human cells deficient in BRCA2 was achieved upon their treatment with inhibitors of PARP1 [98]. This finding led to the development of PARP inhibitors (PARPi) as a novel anti-cancer drug [118]. However, because of the highly adaptive nature of cancer

cells, secondary mutations or reversion mutations of BRCA2 and 1 can lead to PARPi resistance [119, 120]. Given that orthologs of both PARPs and BRCA2 exist in *Dictyostelium*, we wished to characterize the BRCA2 ortholog, to subsequently study whether a synthetically lethal relationship exists with the already characterized PARPs [112]. Most importantly, in the long term, we wished to use the amoeba as a model to study the basis of the synthetic lethal relationship and identify secondary mutations in different genes that could confer resistance to PARP inhibitor (PARPi) treatment of BRCA2 deficient cells.

The aim of this study is to use *Dictyostelium discoideum* as a model to study HR and exploit its advantage over other unicellular models, such as yeast, where key components of DSB repair pathways are not conserved. To this end, through searching the *Dictyostelium* genome database orthologs of known human HR proteins were identified. Importantly, orthologs of RAD51 and BRCA2 were identified. Rad51 is a highly conserved component of HR, whereas BRCA2 was only thought to be conserved in higher eukaryotes. Function of both proteins is central to the HR pathway. My aim was to define whether both proteins are involoved in DSB repair. For Rad51, to prove that the HR pathway shows conservation of function in *Dictyostelium* and the BRCA2 ortholog to demonstrate how *Dictyostelium* is an appealing model for study of HR due to the conservation of proteins that are not found in other lower eukaryotes. The better characterization of the HR pathway will provide an opportunity for the establishment of a reliable, simple model to study HR without the implications and complexity of human cell lines research.

Chapter 2: Materials and methods

2.1 Materials

All buffers were prepared with Millipore Milli-Q double distilled water unless otherwise stated. Buffers were autoclaved at 125^{o}C for 20 minutes or sterile filtered through a 2.2µm filter (Nalgene).

1x SDS PAGE Running Buffer

25mM Tris

250mM Glycine

0.1% SDS

pH 8.3

SDS PAGE Transfer Buffer

50mM Tris

500mM glycine (pH 8.3)

20% Methanol

Enhanced Chemiluminescence reagents

Solution 1: 10mM Tris pH 8.5

0.386mM P-coumaric acid

2.5mM Luminol

Solution 2: 10mM Tris pH 8.5

0.19% H_2O_2

DNA loading dye

0.25% bromophenol blue

40% sucrose

50x TAE

2M Tris Base

50mM EDTA

5.71% 1M glacial acetic acid

pH 8.2

1x SDS Running Buffer (Laemmli buffer)

50mM Tris pH 6.8

20% glycerol

4% SDS

0.2% Bromophenol blue

10% DTT

Store at -20^{o}C

Transfer Buffer

50mM Tris

500mM Glycine (adjusted to pH 8.3)

20% methanol

4x SDS loading buffer (Laemmli buffer)

50mM Tris pH 6.8

20% glycerol

4% SDS

0.2% bromophenol blue

100mM Dithiothreitol

store at -20^{o}C

H50

50mM KCl

20mM Hepes

10mM NaCl

5mM $NaHCO_3$

1mM $NaH_2PO_4.H_2O$

1mM $MgSO_4.7H_2O$

pH adjusted to 7, filter sterilised and stored at -20^{o}C for no more than four weeks.

Tris- Buffered Saline (TBS)

2.48M Tris

137mM NaCl

pH 7.6 and autoclaved.

Chromatin Preparation Buffers

Extraction buffer

50mM Hepes pH 7.5

150mM NaCl

1mM EDTA

1mM Microcystin

1mM NaF

1mM Na_4VO_3

1 protease inhibitor cocktail tablet (Roche) for every 10ml of buffer.

Nuclear Extraction Buffers

Lysis Buffer

50 mM Tris pH 8.0

5 mM MgOAc

10% Sucrose

2%NP40

H_2O

1mM NaF

1mM Na_4VO_3

1µg/ml Microcystin

1 protease inhibitor cocktail tablet (Roche) for every 10ml of buffer.

Use fresh every time

NEB Buffer

20mM Hepes

1M NaCl

5mM $MgCl_2$

0.2mM EDTA

1mM DTT

20% Glycerol

1mM NaF

1mM Na_4VO_3

Microcystin

H_2O

1 protease inhibitor cocktail tablet (Roche) for every 10ml of buffer.

LB agar

1% bactotryptone

0.5% yeast extract

85mM NaCl

1.5% agar

autoclaved

LB Broth

1% bactotryptone

0.5% yeast extract

85mM NaCl

autoclaved

HL5

53mM maltose

1.4% bacteriological peptone

0.7% yeast extract

4mM Na_2HPO_4

3.5mM KH_2PO_4

340µM dihydrostreptomycin sulphate

74µM biotin

82µM Riboflavin

pH adjusted to 6.5 and medium autoclaved.

KK2

3.6mM K_2HPO_4

19mM KH_2PO_4

autoclaved.

SM Agar

1% peptone

56mM glucose

0.1% yeast extract

16mM KH_2PO_4

5.5mM $K_2HPO_4.3H_2O$

4mM $MgSO_4$

1.7% Agar

autoclaved.

Lysis Buffer for fast genomic DNA isolation (LyB)

50mM KCl

10mM Tris pH 8.3

2.5mM $MgCl_2$

0.45% NP40

0.45% Tween 20

autoclaved

before use add 1 µl of fresh Proteinase K (20 µg/ml) for every 25 µl of LyB.

2.2 Methods

2.2.1. General cell culture and genetic manipulations

2.2.1.1 Sequence Alignments

Dictyostelium protein sequences were obtained from DictyBase (www.dictybase.org) and all other sequences from NCBI (www.ncbi.nlm.nih.gov). Protein sequences were aligned with ClustalW and edited in Jalview (http://www.jalview.org/). Jaview was also used to generate phylogenetic trees. Domain search was performed on NCBI using protein Blast (pBlast). Nuclear Localization Signals were predicted using cNSL Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi)

2.2.1.2 Culturing of *Dictyostelium* cells

Dictyostelium (Ax2 and Ax2-derived strains) were grown axenically in HL5 medium at 22^{o}C.

In shaking suspension: cells were cultured in volumes that are a fifth of the flask capacity and shaken at 220 rpm. Cells were maintained at densities between 5 x 10^5 and 5 x 10^6 cells/ml and utilized for experiments at the phase of exponential growth (between 1 and 5 x 10^6 cells/ml). Cell density was determined by counting with a haemocytometer using a Leica DMLS microscope.

In liquid medium plates: Where indicated cells were grown in 90mm or 6-, 12-, 24- and 96-well plates as monolayers and subcultured when confluent. Antibiotic selection was used where necessary, adding to the medium 10µg/ml of blasticidin (Enzo Biosciences), 20 µg/ml or 40 µg/ml of G410 (Sigma) (final concerntration). Plates were incubated at 22^{o}C in the dark.

On solid plates: Cells were grown on SM agar plates covered with a lawn of *Klebsiella aerogenes*.

2.2.1.3 General Cloning

Standard molecular cloning techniques (PCR, restriction enzyme digestion, ligation, TAE gel electrophoresis, phenol-chloroform purification and isopropanol precipitation) were carried out according to standard protocols [121]. Transformation of chemically competent DH5α cells (Invitrogen), mini and maxi preparation of plasmids, PCR product purification and gel extraction

(Qiagen), were performed according to manufacturer's instructions. Sequencing of DNA fragments was performed at Genosys, Oxford, with either custom or universal sequencing primers.

2.2.1.4 Annealing of complementary synthetic oligonucleotides

Oligonucleotides of stock concentration of 100µM where used in a reaction in a final concentration of 10µM along with 50 units of T4 DNA ligase (New England Biolabs) in 1x T4 DNA ligase buffer (NEB). Water was heated in a beaker at 95^{o}C. Reaction tube was incubated at that temperature and left in the beaker until the temperature has dropped to room temperature.

2.2.1.5 Transformation of *Dictyostelium* cells for overexpression

Exponentially growing *Dictyostelium* were harvested and pelleted by centrifugation at 3,000rpm for 3 minutes. For each transfection 5 x 10^{7} cells were used. Cells were then washed twice with 10ml H50 buffer and resuspended in 100µl of H50 prior to mixing with 4-8µg of plasmid DNA (e.g. 4µg of pDXA vector along with 4µg of the pRep helper plasmid). The mixture was added to a chilled 1 mm gap electroporation cuvette (Biorad) and placed in the Gene Pulser XCell (Biorad). Cells were pulsed at 0.65kV with 25µF capacitance with two consecutive pulses and a 6 second recovery between pulses. After 5 minutes of incubation on ice, cells were transferred to a 90mm petri dish containing 10ml of HL5 medium and left to recover for one day. The following day, either 10µg/ml blasiticidin (Enzo Biosciences) or 20µg/ml G418 (Sigma) (final concentrations) were added for selection. The cells were left to select and grow for 7-10 days and when confluent were transferred to shaking suspension and permanent stocks were prepared.

2.2.1.6 Transfection of *Dictyostelium* cells for targeted integration or disruption

The plasmid of interest was constructed using standard molecular cloning techniques. Briefly, two fragments homologous to the sequence flanking the region where the insertion is targeted, were cloned in pLPBLP plasmid on either side of a blasticidin resistance cassette (BsR). The plasmid was amplified in and extracted from transformed *E. coli* DH5α chemically competent cells (Invitrogen) by maxipreparation, according to the manufacturer's instructions (Qiagen). The isolated DNA was subjected to phenol chloroform extraction followed by isopropanol precipitation. Specifically, 1:1 volume of phenol-chloroform is added to the extracted DNA. After vortexing and centrifuging at 14,000g for 10 minutes, the aqueous phase is transferred to a new tube, where 1 volume of chloroform is added. Upon vortexing, the sample is centrifuged at 14000g for 10 minutes. The aqueous phase is then transferred to a new tube and 1/10 of the volume sodium acetate is added, along with 700μl isopropanol. After incubation at -80^{o}C for half an hour, the sample was centrifuged at 14,000g for 15 minutes in a cold centrifuge and the supernatant discarded. Subsequently, 700μl of 70% ethanol was added to the remaining pellet. Centrifugation at 14,000g for 10 minutes followed before the supernatant was discarded and DNA pellet was left to air dry for 15minutes at 65^{o}C. Rehydration of the pellet followed with 20μl of ddH_2O.

Transfection of *Dictyostelium* was performed as described above and 3 serial dilutions of the transfected cells (1:1, 1:10,1:100, 1:1000) were plated on 96-well plates. The following day 10μg/ml blasticidin (final concentration) was added. Cells were incubated at 22^{o}C for 12-15 days.

2.2.1.7 Screening of integration and disruption clones

Surviving colonies derived from 2.2.1.6 protocol, were pooled together in pools of 7 and genomic DNA extracted was screened for the presence of the insertion by PCR. Colonies belonging to pools that gave positive results were individually screened, identified and upscaled to create permanent stocks of the strain containing the insertion. The screening process is described in more detailed below. Genomic DNA was isolated from *Dictyostelium* cells resistant to blasticidin using either Wizard Genomic DNA Purification Kit (Promega) or a fast genomic DNA isolation protocol described below.

2.2.1.8 Fast genomic DNA isolation

Genomic DNA was isolated as described previously [122]. 1 volume of LyB containing Proteinase K is mixed with the pooled cells. After incubating for 10 minutes at 95^{o}C, to inactivate proteinase K, the mixture is ready to be used for PCR. Normally, 5-8μl of the mixture is used to set up a PCR of 25 μl final volume.

2.2.1.9 Cre-loxing of knock-in clones

The blasticidin resistance cassette was removed from successful clones as described previously [123]. Briefly, cells were transfected with pDEX-Cre-NSL following the protocol described in 2.2.1.5. Cells were plated in 96-well plates in 4 different serial dilutions, so that single cell colonies would appear in each well. The following day a final concentration of 20μg/ml G418 (Sigma) was added and cells were left to select for 5-7 days. Medium containing selection was removed and replaced with HL5. Cells were left to grow for another 5 days. Surviving colonies were transferred to a 24-well plate without antibiotics and replica plated in plates containing either 10μg/ml blasticidin (Enzo Biosciences)

and 20μg/ml G418 (Sigma) (final concentration). Clones sensitive to both antibiotics were then transferred to Ka plates. After genomic DNA isolation and PCR screening for the loss of the BsR cassette, successful clones were scaled up and permanent stocks were made.

2.2.1.10 Preparing permanent *Dictyostelium* stocks

5 x 10^7 cells were pelleted by centrifugation at 3000 rpm for 3 minutes. Cells were resuspended in 1ml FA (50% Fetal Calf Serum, 50% HL5 and 5% DMSO). The mixture is places in Cryotubes (Fisher Scientific) which are then gradually cooled down to -80^{o}C.

Cells were resurrected from -80 ^{o}C every week by scraping a small amount of the frozen cell-medium mixture and placing it on a SM agar plate covered with Ka. The cells were left to grow for 3-4 days. Subsequently, the leading edge of a *Dictyostelium* plaque was picked with a sterilized loop and resuspended in 10ml HL5 containing 10 units of penicillin and 10 μg/ml of streptomycin, to kill bacteria transferred in the plate along with *Dictyostelium* cells. The following day selection was added, as described above, where necessary.

2.2.3 Phenotypic studies

2.2.3.1 Whole Cell Extract (WCE) preparation

The desired number of cells (most commonly 5 x 10^6 cells) were pelleted by centrifugation at 3,000 rpm for 3 minutes and resuspended in 1x SDS loading buffer. The resulting mixture was boiled at 100^{o}C for 7 minutes.

2.2.3.2 Cellular fractionation

Subcellular fractionation was achieved as described previously [124]. Briefly, cells grown exponentially were seeded at 5 x 10^6 cells/ml and treated with phleomycin (Sigma) or mock treated. At specific time points, cells were pelleted by centrifugation at 3,000rpm for 3 minutes and washed with 1ml of ice cold KK2. Subsequently, cells were resuspended in extraction buffer supplemented with 0.1% Triton X-100 (Sigma) (200µl of buffer for every 6 x 10^6 cells), incubated at 4^{o}C for 15 minutes and centrifuged at 14,000g for 3 minutes. The process was repeated and the resulting supernatant (S1) was either discarded (for experiments where only the chromatin fraction was subsequently used for western blot analysis) or kept and then precipitated with 100% acetone overnight*. Finally, the remaining pellet (P2) was resuspended in extraction buffer supplemented with 20µg/ml RNAaseI (Sigma). Samples were incubated at room temperature, with agitation for 30 minutes. Centrifugation at 14,000g for 3 minutes followed. Where necessary, an extra extraction step was performed where the P2 pellet is resuspended in extraction buffer supplemented with 300µg/ml DNAseI and 5mM $MnCl_2$. The samples were then incubated at 37^{o}C for 30 minutes. Following centrifugation at 14,000g for 3 minutes the supernatant (S3) was separated from the pellet (P3) and precipitated with acetone*. All pellets resulting from supernatant precipitation (S1, S2, S3) as well as insoluble pellets (P2,P3) were resuspended in 1x SDS loading buffer. Equal cell number equivalents were loaded on SDS PAGE gels of appropriate percentage and analysed by western blotting.

*Precipitation was achieved when six volumes of acetone were added to the extract and mixed gently. The acetone – extract mixture was kept at -20^{o}C overnight. The following morning, samples were centrifuged at 14,000g for 15 minutes. Upon centrifugation, the supernatant was discarded and pellet resuspended in 60µl of 1x SDS loading. Samples were then boiled for 7 minutes at 100^{o}C buffer.

2.2.3.3 Preparation of nuclear extracts

2 x 10^{8} exponentially growing cells were centrifuged at 3,000 rpm for 3 minutes and the supernatant discarded. Subsequently, cells were washed with 1ml ice cold KK2 and centrifuged at 3,000 rpm for 3 minutes. Supernatant was discarded and 1ml of lysis buffer added. The lysate was then incubated at 4^{o}C agitating for 10 minutes and centrifuged at 5,000 rpm for 5 minutes. Subsequently, the supernatant was discarded and the remaining pellet (the nuclei) resuspended in 100µl of Nuclear Extraction Buffer with a 26G needle. The lysate is incubated at 4^{o}C, with agitation, for 30 minutes and centrifuged at 13,000 rpm for 5 minutes. The supernatant was transferred to a new tube and resuspended in 4x SDS loading buffer along with 10% DTT. Samples were boiled at 100^{o}C for 7 minutes. Equal amounts of extract were analysed by western blotting.

2.2.3.4 Western blotting

Samples were run in a Mini Protean II chamber (BioRad) in appropriate percentage SDS-PAGE gels, alongside protein size markers (both pre-stained and unstained, depending on the occasion) and subsequently transferred to either nitrocellulose (Fisher) or Polyvinylidene fluoride (PVDF) (Millipore) using

the Mini Protean II transfer chamber and gel running apparatus (BioRad), at 250mA for 2 hours. Membranes were blocked in blocking buffer (1x TBS, 0.2% Tween, 5% non-fat milk powder) for 1 hour, in the case of nitrocellulose or bocked with 100% methanol and dried in the case of the PVDF membrane. Subsequently, membranes were incubated with primary antibody in the appropriate dilution of blocking buffer, either for an hour at room temperature or overnight at 4^{o}C. Washing with TBS and 0.2% Tween (wash buffer) followed. The wash was repeated another three times for 10 minutes each time. Membranes were then incubated for an hour at room temperature, with the relevant Horseradish peroxidase (HRP)-conjugated secondary antibody in blocking buffer, at room temperature (RT). After the incubation was finished, the membranes were washed 4 times, for 10 minutes each time, shaking, with wash buffer. Finally, membranes were exposed to a mixture composed of 1ml of each of the enhanced chemiluminescent reagents for half a minute. Light emission from the membranes was captured on photographic film, which is then developed to visualise the western blot result.

2.2.3.5 Treatment of cells with auxin

Cells were treated with various concentrations of 1-Nauthalenacetic acid (NAA), an artificial form of auxin (0.25-0.5-1mM) for various times (as indicated) or mock treated with water. Subsequently, cells were used to either prepare whole cell extracts or nuclear extracts, trypan blue viability assay and sensitivity to phleomycin, using the methods described.

2.2.3.6 Sensitivity assay upon Auxin treatment

This assay is based on the method initially used by *Deering et al.* [108]. In this case, the experiment was performed using phleomycin (Sigma) as a damaging agent, at a various concentrations. Cells are seeded at a density of 1 x10^6 cells/ ml, in a final volume of 4ml in small conical flasks. Cells were pre-treated with 0.5mM auxin or mock treated for four hours at 22^{o}C, shaking in the dark. Subsequently, phleomycin is added to cells at increasing concentrations as indicated for 1 hour. When the treatment is finished, 10µl of each sample are mixed with 990µl of KK2 (10^4 cells/ml). A mixture of KK2 and *Klebsiella aerogenes (Ka)* is prepared. 140mm SM agar plates were also prepared either plain or containing 0.5mM auxin. For each sample, two SM agar plates were spread as follows: 350µl of KK2-Ka are mixed on a plate with 25µl of cell mixture (approx. 200 cells). The mixture is then spread with a sterilised spreader on the plate. Plates were incubated at 22^{o}C for three days. On the third day, *Dictyostelium* plaques start appearing. Colonies were counted for three consecutive days and scored relative to the zero control. A total of three experiments are then combined to allow statistical analysis of results.

2.2.3.7 Cell viability assay using Trypan Blue staining

This assay is based on the ability of live cells to exclude the Trypan blue dye. Dead cells have permeable membranes, therefore stain blue after incubation with the dye. Trypan blue was added to a small volume (10µl) of cells in a 1:1 ratio. The final concentration of the dye in the mixture is 0.1%. Cells were then incubated with the dye for 15-20 minutes. Subsequently, 10µl of the mixture were mounted on an objective (Fisher Scientific) and covered with a 0.1mm coverslip (Fisher Scientific). A total of 200 cells were counted. The number of

alive and dead cells were scored and percentages of dead cells relative to the control cells were calculated and then plotted in a graph.

Chapter 3: Homologous Recombination-mediated repair in *Dictyostelium.*

3.1 Introduction

Homologous Recombination (HR) is a key process for the maintenance of genome integrity conserved throughout evolution, from bacteria to humans. HR is utilized to repair DNA Double Strand Breaks (DSBs), the most toxic type of DNA lesion. Upon processing of the broken DNA ends by various nucleases, and generation of extensive ssDNA threads, a nucleoprotein filament is formed that will perform homology search and strand invasion. In bacteria, this function is performed by Rec-A recombinase [125]. Rad51, is its eukaryotic counterpart, a highly conserved protein that has been well studied in various organisms since it was first identified in yeast [50, 126]. As described in Chapter 1, although the strand invasion step is essential for the repair process, the end processing step preceding the strand invasion and repair intermediates resolution, are equally important and have been the subject of numerous studies over the years [36].

Although some components of the machinery are highly conserved throughout evolution, others do not appear in the genome of lower eukaryotes or have slightly different functions. For example, Rad52 is a mediator of Rad51 and has been shown to be essential for DNA repair in yeast, as Rad52 mutated cells show extreme radiosensitivity when treated with IR [127]. Surprisingly, however, depletion of Rad52 in vertebrate cells has little to no effect on recombination repair [60]. This pronounced difference is attributed to the existence of BRCA2, which, in vertebrate cells, functions to regulate Rad51 localization by displacing RPA from ssDNA and promoting Rad51's association [61]. Experiments in

vertebrate cells where Rad51 paralogs were mutated in a *brca2*$^{-/-}$ background show that mutation of most Rad51 paralogs causes a mild increase of radiosensitivity upon IR treatment [128]. Strikingly, in the case of Rad52 a synthetically lethal phenotype is observed [128, 129]. From the above, it becomes clear that Rad52 does act as a mediator of Rad52, but its role is not as central as in yeast, making it hard to transfer findings from yeast to humans.

Most unfortunate is the fact that some of the components deemed to have central roles in DNA damage signaling in human cells, are absent from lower eukaryotes that are used as models for DNA repair (e.g. *S.cerevisiae*). The most prominent example are Poly-ADP Ribose Polymerases (PARPs), molecules that play a central role in signaling different types of damage in eukaryotic cells [130]. Additionally, DNA-PKcs, a PIKK kinase important for DSB damage signaling and repair is also not present in yeast [2].

Apart from the absence of key NHEJ components, a central component of human HR repair is not present in yeast. The tumor suppressor BRCA2 has a central role in HR, where it acts as an essential mediator of Rad51[73-75]. However, no orthologs of this protein can be found in yeast. Instead, in *S.cerevisiae* Rad52 plays a role similar to that of BRCA2, serving as a Rad51 mediator protein [131]. Finally, the Fanconi Anemia pathway is underrepresented in yeast, as only FANCM is conserved [113]. All the above constitute a few examples of many cases, which have lead to the conclusion that although yeast has served as a good model for identification of basic components of DNA repair mechanisms, several of the results obtained do not correlate to human DNA repair.

Dictyostelium discoideum has long been used as a model organism for the study of various cellular processes including chemotaxis and phosphotyrosine signaling as well as cell differentiation [100, 105]. The genome of the organism has recently been sequenced and an online database created (www.dictybase.org [104]). Through bioninformatic analysis and experimental validation, we have been able to identify orthologs of DNA repair proteins implicated in DSB repair pathways that are not conserved in other models (e.g. budding yeast). Orthologs of DNA-PKcs and Artemis, which are implicated in NHEJ, [109, 110] as well as orthologs of the damage sensors, PARPs [112] have been identified. Finally, a minimal FA pathway has been identified in *Dictyostelium* [113]. These findings make *Dictyostelium* an attractive model to study DNA repair pathways, due to the high conservation of human DNA repair components, which cannot be observed in other lower eukaryotes, but also its simplicity as a model organism.

Since *Dictyostelium* shows surprisingly good pathway conservation regarding DSB repair pathways such as NHEJ, I wanted to more thoroughly test conservation of another major DSB repair pathway, HR. To determine the degree of conservation of the pathway, I initially conducted bioinformatic searches querying the *Dictyostelium* genome database for known components of the HR pathway in humans. Putative orthologs of most known, highly conserved HR proteins were identified. Interestingly, we were able to identify one protein (DDB0306020), which seems to be a putative ortholog of the human BRCA2 protein. All the above findings prompted me to test whether *Dictyostelium* would be a good experimental model to study HR. To achieve

this, I conducted experiments to determine whether the most conserved and important factors for HR repair, the Rad51 proteins, display a conserved function in this organism. Through biochemical fractionation of chromatin upon induction of DSBs, I was able to observe that Rad51 is indeed recruited to chromatin upon DSB induction, in a time dependent manner. At the same time I compared the kinetics of recruitment of Rad51 to Ku80, a NHEJ factor, aiming at understanding the relationship between two different DSB pathways. Taken together these data show that Rad51 gets recruited to chromatin later than Ku80. All the above indicate how *Dictyostelium* could serve as a good model to study HR.

3.2 Results

3.2.1 Key components of Homologous Recombination-mediated repair are conserved in *Dictyostelium discoideum.*

I conducted a search querying the *Dictyostelium* genome database (www.dictybase.org) for orthologs of human HR proteins. The sequence of the human protein of interest was obtained from NCBI. Subsequently, using the BLAST tool available on http://dictybase.org/tools/blast I queried the database of putative proteins for homology with the given sequence. In the cases where positive results were obtained, the retrieved protein sequence was inserted in the NCBI BLAST (http://blast.ncbi.nlm.nih.gov/) tool to cross reference the finding with already known and described orthologs of the protein in question. Wishing to compare conservation with that of another lower eukaryote, the budding yeast *Saccharomyces cerevisiae*, I was able to identify several components that are conserved between the three organisms that are known to function at different stages of the repair process (Table 3.1).

Importantly, the key sensor of DSBs are conserved. More specifically, two key components of the MRN complex (MRX in budding yeast), Mre11 and Rad50, are present in the *Dictyostelium* genome. Nbs1/Xrs2, the third component of the complex, which has been shown to interact with CtIP/Ctp1/Sae2 in humans and yeast to promote end resection and HR [42], is seemingly absent in *Dictyostelium.* The phosphatidylinositol 3-kinase ATM, which it recruits to sites of damage via direct interaction with Nbs1 is also absent [132]. At the same time, a histone variant found to be deposited and phosphorylated in the vicinity of DSB [5], histone H2AX, is also conserved, supplying a way to monitor DSB induction in *Dictyostelium*. γH2AX is also very important to trigger events downstream of damage recognition via recruitment of repair proteins to the site of damage.

A critical step for the repair process to be channelled towards HR is the processing of the damaged DNA ends. The exonuclease activity of Mre11 as well as CtIP/Ctp1/Sae2, have been shown to be important for this step [42, 133, 134]. For extensive DNA end resection Exo1 and Dna2 are also required [48]. *At* the same time, the helicases BLM, WRN and their yeast orthologs Sgs1 play an important role along with topoisomerase 3 in assisting the role of EXO1/Exo1 and DNA2/Dna2 by unwinding DNA at the break site (TOP3) [41-43, 48]. We were able to identify putative orthologs of all the above proteins in *Dictyostelium*.

H.sapiens	*D.discoideum*	*S. cerevisiae*	Function
H2AX	H2AX	H2AX	Histone variant that gets phosphorylated upon DSB induction
ATM	-	Tel1	PIKK family kinases / sensors of DNA damage phosphorylate downstream targets
ATR	Atr1	Mec1	
Nbs1	-	Xrs2	Partner of Mre11
Rad50	Rad50	Rad50	Tethering of DNA ends, partner of Mre11
Mre11	Mre11	Mre11	DNA end resection and DNA damage checkpoint activation
RPA	rfa1	rfa	Coating and stabilisation of ssDNA
Exo1	Exo1	Exo1	DNA end resection
CtIP	Sae2	Sae2	End resection and pathway choice
53BP1	DDB0305622	Rad9	DNA damage checkpoint and DSB repair pathway choice
DNA2	Dna2	Dna2	Helicases implicated in end resection before HR
WRN	wrn	Sgs1	
BLM	blm		
TOP3	Top3	Top3	Topoisomerase involved in HR acting together with BLM and WRN
SLX1/SLX4	-	Slx1/Slx4	Holliday Junction Resolvases
GEN1	-	Yen1	
MUS81	Mus81	Mus81	

RAD51	*rad51-1, rad51-2*	Rad51	Homology search and strand invasion
RAD52	*rad52*	Rad52	Mediator of Rad51
RAD54	*rad54*	Rad54	Dissociation of D-loop upon completion of DNA synthesis
Rad51C	DDB0232315	Rad55, Rad57	Rad51 paralogs that interact with Rad51 and stabilise the Rad51-ssDNA nucleoprotein filament
Rad51D	DDB0232317		
XRCC3	*xrcc3*		
XRCC2	*xrcc2*		
BRCA2	DDB0306020	-	Scaffold and stabiliser of Rad51

Table 3.1: Most proteins implicated in Homologous Recombination in humans mediated repair of DSBs appear to be conserved in *Dictyostelium* but not in yeast. In the above table, most of the proteins required or implicated in HR in humans are listed. All the *Dictyostelium* equivalents can be found in the second column. In the third column, the equivalents from budding yeast are listed.

Once the ends have been processed, they are coated by RPA (Replication Protein A), which attracts ATR (ATM and Rad3-related) PIKK family kinase, which activates the DNA damage checkpoint [135]. At the same time, Rad51 displaces RPA and coats the processed DNA ends to form a nucleoprotein filament, a reaction mediated by the essential factor BRCA2 [51, 136]. Several Rad51 paralogues (XRCC2, XRCC3, Rad51C, Rad51D, Rad51B in humans; Radd55, Rad57 in yeast) have been shown to be essential for the stabilisation of the nucleoprotein filament. The repair process commences when the Rad51 filament performs a search for a homologous sequence, which is then invaded for the formation of a D-loop (Displacement loop) aided by Rad54 motor protein and Rad52 [63, 131]. A search for orthologs of all the above in *Dictyostelium* shows potential conservation for all the above mentioned proteins (Table 3.1). When the Rad51B sequenced was used to search the genome for an ortholog, several homologous proteins appeared. In this case a putative ortholog potentially exists, but could not be specifically determined.

Upon strand invasion, DNA synthesis using the homologous sequence as a template and the invading strand as a primer is mediated by DNA polymerases. Different events taking place after D-loop formation and extension of the 3' end of the invading strand, can lead to repair through different sub-pathways. In the case of SDSA (described in chapter 1), the extended 3' invading strand then dissociates assisted by Rad54, to anneal to the second end of the break and prime DNA synthesis at the non-invading strand of the break to complete the repair process. In the case where a double holliday junctions (dHJ, a four stranded repair intermediate) is formed via capture of the second end of the break by the D-loop, the action of structure-specific endonucleases MUS81-

EME1, GEN1 and SLX4/SLX1 is required for HJ resolution [38]. Of those, only a MUS81 ortholog was identified in *Dictyostelium.* Alternatively, for the dissolution of a dHJ, a procedure mechanistically distinct from the resolution process (see chapter 1), the actions of BLM helicase and Topoisomerase III are required. These two proteins appear to be conserved in *Dictyostelium*.

It is interesting to observe how several of the components and their functions mentioned above are conserved in *Dictyostelium,* but are either absent or underrepresented in budding yeast. Such examples are all Rad51 paralogs, where in yeast only Rad55 and Rad57 exist instead of the 5 human paralogs. Also, the functions of BLM and WRN helicases are combined in Sgs1. Most importantly, BRCA2, a protein which was previously thought to only exist in higher eukaryotes and is essential for proficient Homologous Recombination in various organisms, seems to be conserved in *Dictyostelium* [74, 86-90].

These findings are summarized in table 3.1 and suggest very high conservation of HR in the amoebae, which could potentially serve as a good model for the study of the HR pathway. A good indication that this might be the case is given by the study of an Exonuclease 1 mutant in *Dictyostelium,* which is deficient in homologous recombination and hypersensitive to DSB inducing agents [110].

3.2.2 A subcellular fractionation protocol for monitoring DNA repair proteins' recruitment to damaged chromatin.

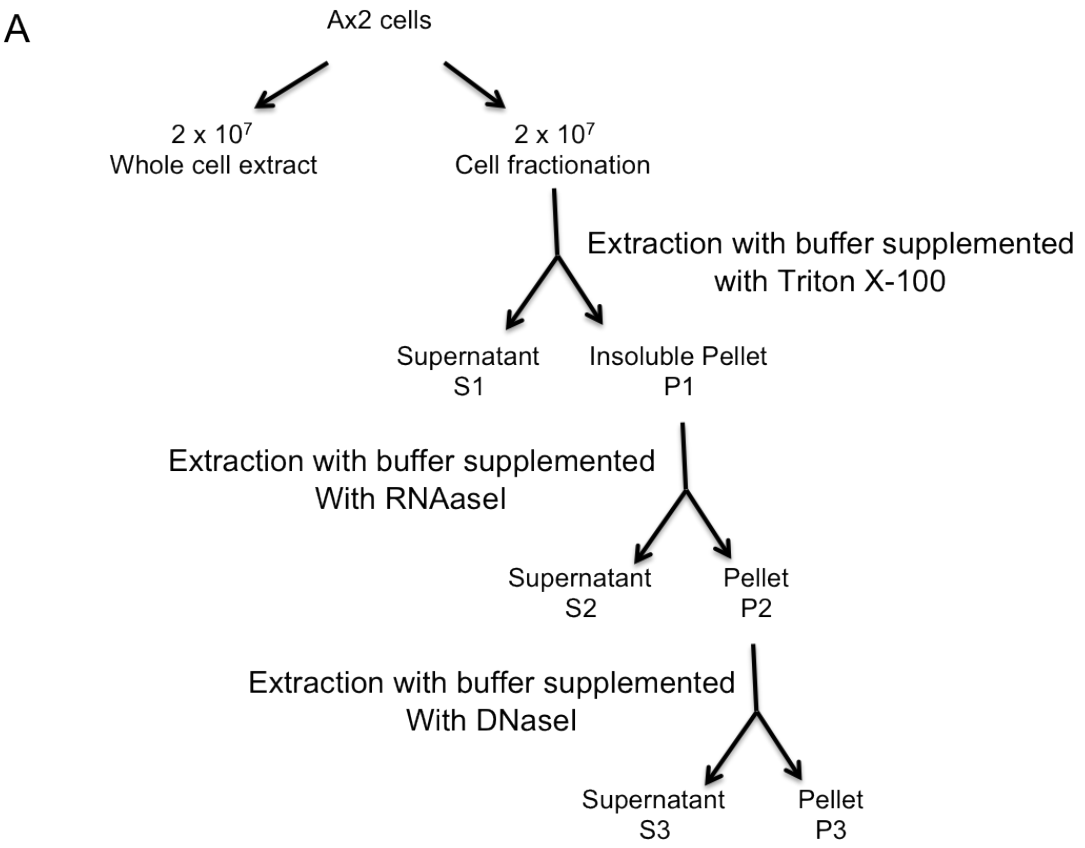
After identifying putative orthologs of most major HR proteins, I wanted to experimentally test the role of HR proteins in DNA repair in *Dictyostelium*. Experiments conducted in vertebrate and yeast cells illustrate that NHEJ

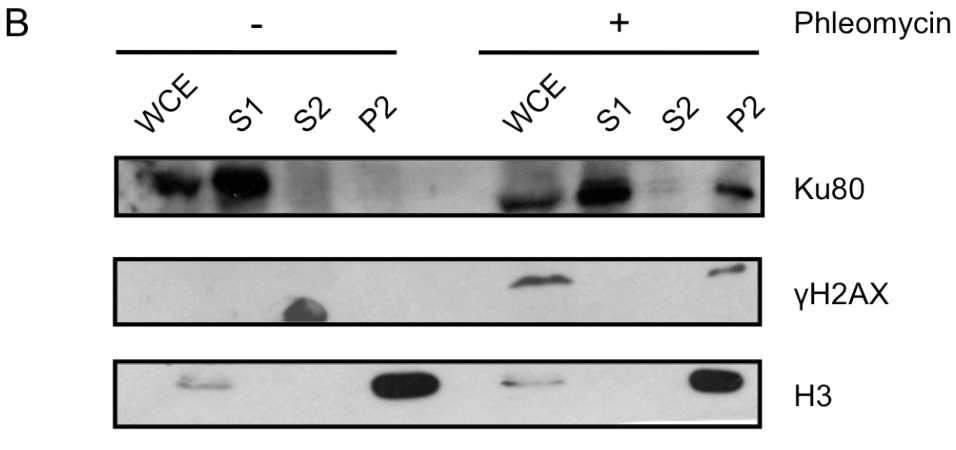
proteins are recruited to chromatin and the repair process completed faster than for HR [39, 137, 138]. The observation is mainly attributed to the need for end processing and the generation of a specific substrate for HR proteins to be recruited at the damage site. Experiments in human and *Dictyostelium* cells suggest that NHEJ components get recruited at the site of the break first during G_2 phase to attempt DSB repair. If NHEJ fails to repair the break, HR is utilized and end processing is promoted [110, 139]. The above observation is based on genetic studies conducted in *Dictyostelium* [110]. To verify this hypothesis with biochemical data I directly compared the kinetics of recruitment to chromatin of key representatives of each repair pathway upon DSB induction.

It is very common that the specific enrichment of DNA repair proteins is observed via indirect immunofluorescence. However, immunofluorescence can prove to be problematic in some cases. For example, in the case of Ku80, when indirect immunofluorescence was employed DNA damage induced foci were undetectable in the nucleus, although it is well established that NHEJ proteins get recruited to chromatin upon damage. This is attributed to the very fast recruitment and dissociation of the complex at DNA breaks, as well as the 1:1 stoichiometry of DNA end : Ku [140]. To circumvent this problem, I applied an assay previously described in mammalian cells [124] to *Dictyostelium.* The assay allows for monitoring of the gradual accumulation of DNA repair proteins on chromatin upon damage induction. More specifically, cells are initially treated with a DSB inducing agent and then subjected to subcellular fractionantion. This allows for visualization of the specific enrichment of DNA repair proteins in the chromatin enriched insoluble pellet that is the end product of several extraction steps (see Figure 3.1). Using the same method, we were able to observe

increasing enrichment of repair proteins dependent on time and dose of the damaging agent.

Initially, I sought to validate this method by assaying enrichment of a known DSB repair protein. Ku80 is a component of the NHEJ repair pathway and has been shown to be important for repair of DSB in *Dictyostelium* [109]. To determine subcellular localization of Ku80, cells were treated with phleomycin and subsequently subjected to subcellular fractionation as described in figure 3.1A. All three fractions, as well as whole cell extracts, were subject to western blotting with a Ku80 antibody. Histone H3 serves as an indicator of equal loading of the insoluble fractions, as well as the presence of chromatin. Histone γH2AX is used as a marker of DSBs [5]. In the absence of damage, Ku80 is only found in S1, the soluble fraction resulting from detergent extraction of cells. DSB induction by phleomycin, caused a clear enrichment of Ku80 in P2, the insoluble fraction resulting after two successive extraction steps (Figure 3.1B). The above suggest that Ku80 is recruited to chromatin upon damage.


A
Ax2 cells
2 x 10^7
Whole cell extract
2 x 10^7
Cell fractionation
Extraction with buffer supplemented
with Triton X-100
Supernatant
S1
Insoluble Pellet
P1
Extraction with buffer supplemented
With RNAaseI
Supernatant
S2
Pellet
P2
Extraction with buffer supplemented
With DNaseI
Supernatant
S3
Pellet
P3


B
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WCE
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P2
Ku80
γH2AX
H3

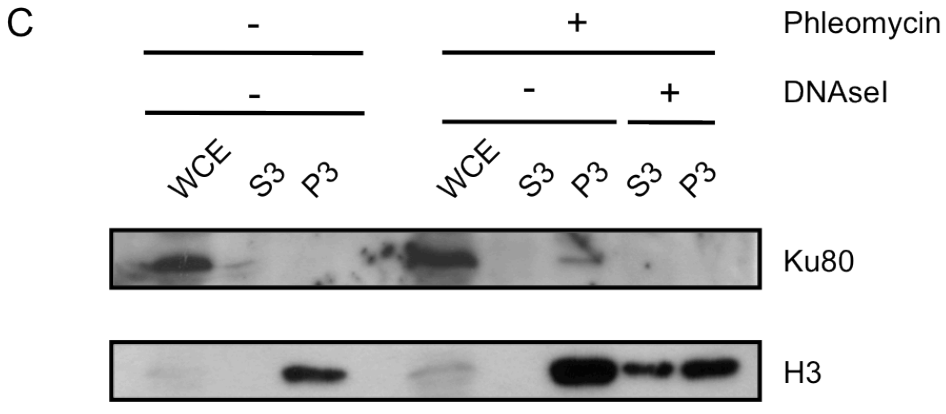

C
-
+
Phleomycin
-
-
+
DNAseI
WCE
S3
P3
WCE
S3
P3
S3
P3
Ku80
H3

Figure 3.1: Ku80 is enriched on chromatin upon Double Strand Break induction (A) Schematic representation of the cell fractionation experiment. Cells are first lysed with 0.1% triton. Upon centrifugation, fractions S1 (soluble) and P1 (insoluble pellet) are obtained. The insoluble fraction (P1) is then subject to a further extraction step with buffer supplemented with 200µg/ml RNAaseI. After this extraction step the insoluble pellet constitutes the "chromatin enriched" fraction (P2) and a soluble fraction is collected (S2). Where necessary, the pellet is subject to one final extraction step, where it is treated with DNaseI. This results in the isolation of a soluble (S3) and insoluble fraction (P3). Whole Cell Extracts (WCE) are prepared by lysis in Laemmli buffer. All soluble fractions were precipitated with 6:1 volumes of 100% acetone. Precipitants were then resuspended in 1x SDS loading buffer and boiled at 100^{o}C for 7 minutes, as were the insoluble pellets. The protocol is described in detail in materials and methods. (B) Ku80 is enriched in a detergent extraction resistant fraction upon administration of Double Strand Breaks and cell fractionation. Exponentially growing Ax2 cells were seeded at 5 x 10^6 cells/ ml and when indicated treated with 200µg/ml of phleomycin for one hour. A total of 2 x 10^7 cells were used. Upon treatment, cells were washed, pelleted and used to either prepare WCE, or subject to fractionation with two consecutive extractions as described in (A). Extracts of both untreated (left) and treated with phleomycin (right) cells are shown. An equivalent of 5 x 10^6 cells were loaded in each of the lanes. Protein samples were then subject to SDS PAGE electrophoresis (10% gel for Ku80 and 15% for γH2AX and H3), followed by liquid transfer on PVDF membrane. Membranes were blotted with the antibodies indicated. Antibody against histone H3 is used to confirm equal loading of the fractions. γH2AX is used as an indicator of DSBs and of the chromatin fraction. (C) Enrichment of Ku80 in the detergent extraction insoluble fraction is chromatin specific. Extraction process was as described in (A), including treatment with 300µg/ml DNaseI, resulting in supernatant S3 and pellet P3. Samples were analysed as in (B).

To verify that the enrichment observed is DNA-dependent, we performed the procedure as described above, this time subjecting the pellet P2 to a further extraction step with buffer supplemented with DNaseI. DNaseI treatment fragments DNA, rendering it soluble along with the proteins associated with it. Importantly, in the absence of damage and when extracts are treated with buffer that does not contain DNaseI, no enrichment of Ku80 can be observed. As expected, when phleomycin treated cells are extracted in the absence of DNaseI, Ku80 is enriched in the insoluble fraction (P3). However, when the extract is treated with DNAseI, Ku80 is lost from P3 (Figure 3.1C). It is obvious that fraction S3 contains solubilised chromatin, as indicated by the presence of histone H3 in the fraction. Unfortunately, because the fragmentation of the chromatin is not complete, we can still detect H3 in the P3 lane of the damaged cells derived extract treated with DNaseI. We suspect that the partial fragmentation of the chromatin in this experiment has reduced the Ku80 signal in the P3 fraction, but does not allow the accumulation of enough protein molecules in the S3 fraction for us to be able to obtain a clear signal coming from that fraction. Nevertheless, we consider this data indicate that the enrichment of Ku80 in the P3 fragment is chromatin dependent.

From the above, we can conclude that when DSBs are administered to *Dictyostelium* cells, Ku80 gets specifically associated with chromatin, an association that is absent when no damage is administered to the cells and the majority of the protein is cytoplasmic. To further validate and refine the system I tested whether Ku80 levels increased in the chromatin fraction with increasing time of treatment and dose of the damaging agent.

3.2.3 Ku80 is recruited to chromatin in a dose- and time-dependent manner upon DSB induction.

Having established that the enrichment of Ku80 in the detergent insoluble fraction is DNA-dependent, I next examined whether the amount of Ku80 getting recruited to DNA is increased along with the number of DSBs induced.

Ax2 cells were treated with increasing doses of phleomycin up to 400μg/ml. Cell fractionation followed. Importantly, more Ku80 is recruited to chromatin as the doses of phleomycin increase (figure 3.2A, top panel). The histone γH2AX signal is an indicator of the increasing amount of damage. Based on the above experiment, 200μg/ml of phleomycin will be used for all subsequent experiments, as an intermediate dosage that allows us to observe recruitment of DNA repair proteins without reaching saturation.

Using the above indicated dose, I next examined the recruitment of Ku80 on chromatin at different time points upon DSB induction. Cells were treated with phleomycin for various times up to 180 minutes and then extracted. The chromatin enriched pellet was analyzed by western blotting with the indicated antibodies. Importantly, Ku80 gets recruited to chromatin after only half an hour of treatment with the damaging agent and reaches a saturation point after an hour (Figure 3.2B). Again, to illustrate how the recruitment to chromatin is due to damage induction and not to increased expression levels, the same analysis was performed with whole cell extracts (Figure 3.2B).

From the above, it becomes clear that the association of Ku80 with chromatin is directly proportional to the amount of breaks administered to the cells. At the

same time, the recruitment of the protein is rapid and reaches a saturation point after 1 hour of damage administration.

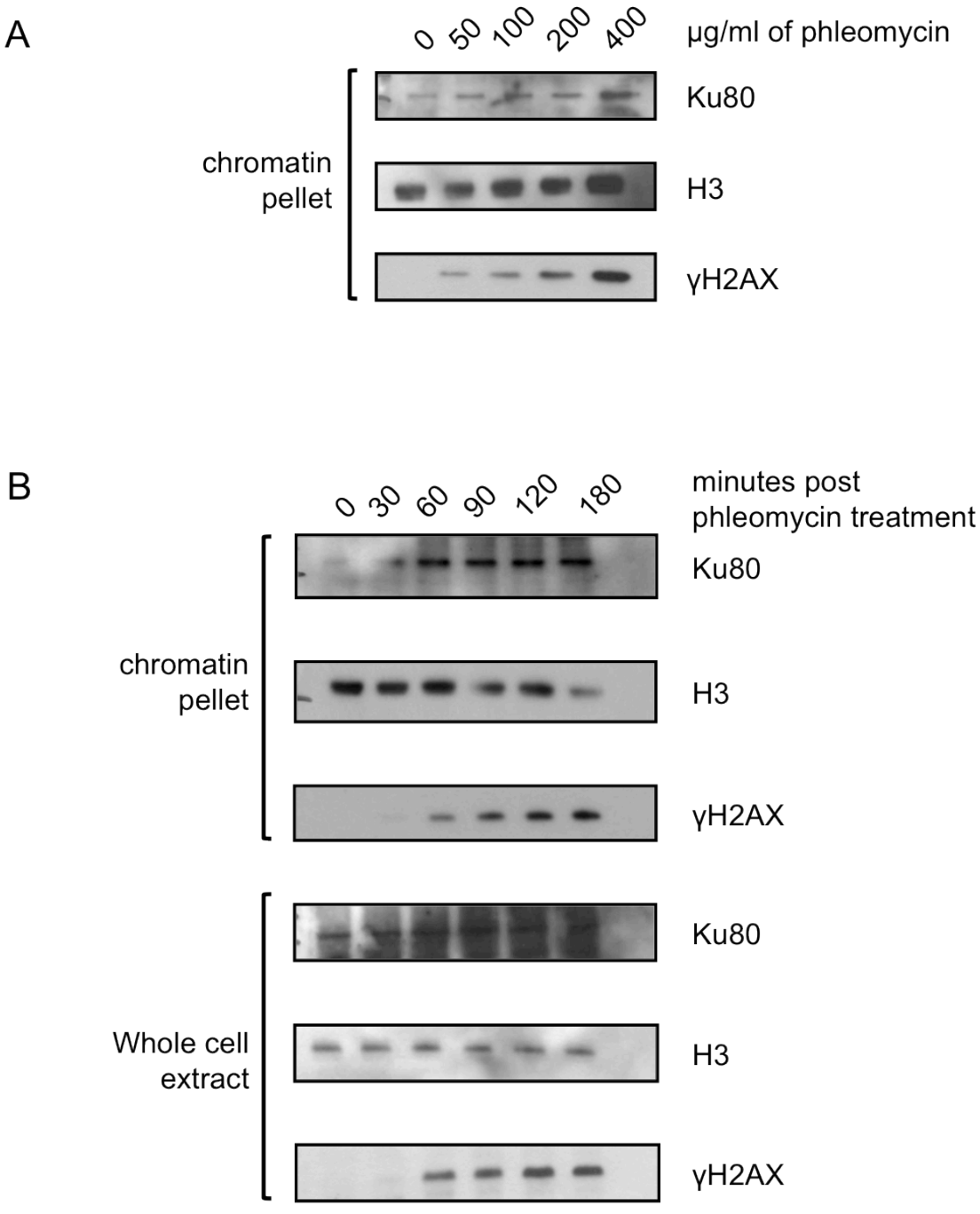

A
0
50
100
200
400
µg/ml of phleomycin
chromatin pellet
Ku80
H3
γH2AX
B
0
30
60
90
120
180
minutes post phleomycin treatment
chromatin pellet
Ku80
H3
γH2AX
Whole cell extract
Ku80
H3
γH2AX

Figure 3.2: Ku80 gets recruited to double strand breaks in a dose and time-dependent manner. (A) Exponentially growing Ax2 cells were exposed to the indicated doses of phleomycin, up to 400µg/ml. Subsequently, cells were used for cell fractionation. Extracts were analysed by SDS PAGE electrophoresis and western blotting with the indicated antibodies. (B) Exponentially growing Ax2 cells were treated for up to 180 minutes with 200µg/ml of phleomycin at a density of 5 x 10^6 cells/ml. Generation of Whole Cell Extracts or cell fractionation (top panel). Extracts were analysed by SDS PAGE electrophoresis and western blotting with the indicated antibodies.

3.2.4 Rad51 is recruited to chromatin with kinetics similar to Ku80

Having established a technique to monitor the kinetics of recruitment of DNA repair proteins to chromatin, I wanted to utilize it to compare recruitment of HR to NHEJ proteins. The Rad51 recombinase, an essential component of the HR machinery was chosen as a representative of the pathway. Rad51 is essential for HR, as it forms a nucleoprotein filament with the long ssDNA tracts that result from extensive end processing of the broken DNA at DSB sites. Homology search follows and the nucleoprotein filament then invades the homologous sequence forming a D-loop to prime DNA synthesis, the initial step of HR repair [50, 53, 57].

In *Dictyostelium*, Rad51 (DDB0304849) is a 351 amino acid long protein expressed by two identical genes, *rad51-1* and *rad51-2*, which probably resulted from gene duplication. Strikingly, the *Dictyostelium* Rad51 protein shows 74% conservation in its overall sequence and 79% identity in its catalytic domain, when compared to the human ortholog. Comparing it to other Rad51 orthologs, a percentage identity ranging from 55-73% can be observed (Appendix C, Figure S1 and Table S1).

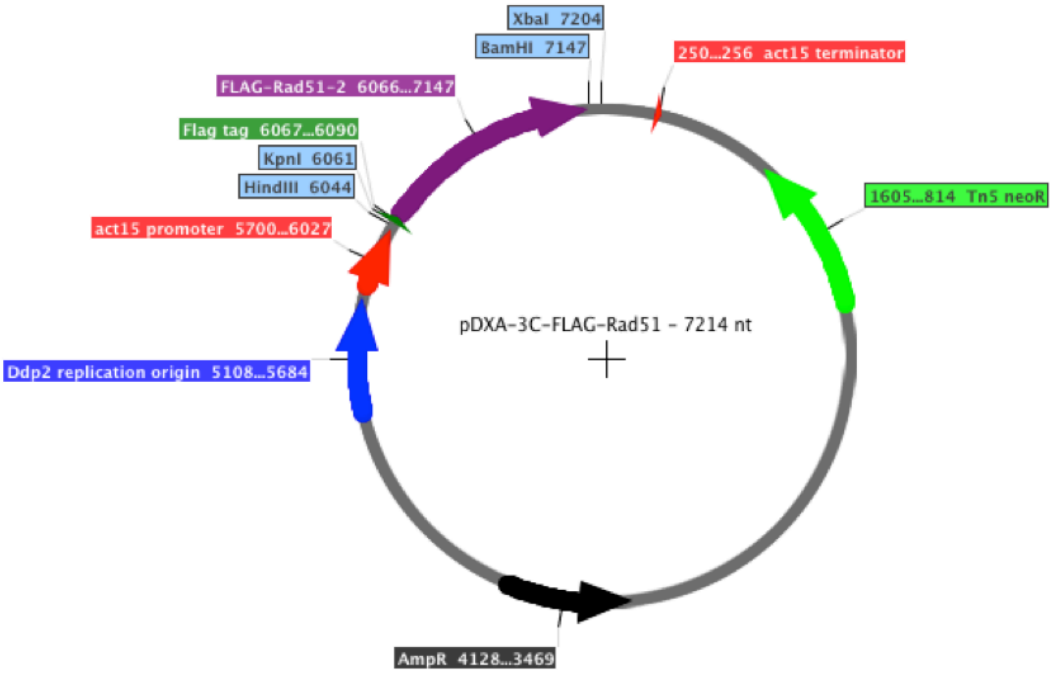
For the purposes of these experiments, a plasmid to drive the expression of recombinant, epitope-tagged *rad51-2* was introduced into Ax2 cells. More specifically, the sequence encoding the *rad51-2* gene, was cloned from cDNA by Polymerase Chain Reaction (PCR) and inserted, along with a FLAG tag, into the pDXA-3C expression vector [141] (Figure 3.3A). The cloned gene is under the control of the *Dictyostelium* actin 15 promoter and hence, is constitutively expressed once transfected into cells. It is necessary that the construct is

transfected along with the pREP helper plasmid, which will allow it to replicate independent of the nuclear DNA. It is also necessary for G418, a neomycin analogue, to be added to the growth medium, so that the plasmid is retained by the cells in subsequent cell divisions. The construct was checked for the presence and size (1056bp) of the insert by restriction digestion, (Figure 3.3B) and sequenced. Subsequently, *rad51-2*-pDXA3C was electroporated into Ax2 cells. A control strain, transfected with empty pDXA-3C vector was also generated. Upon transfection, cells were grown in 90mm petri dishes containing HL5 and cells surviving G418 selection formed a pool of transformants that was subsequently used for experiments.

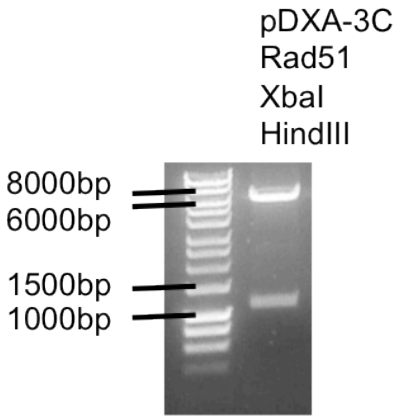
Whole cell extracts were prepared from exponentially growing cells of both strains and used to verify the expression of the recombinant protein by Western blotting using an antibody against the FLAG epitope. The FLAG antibody recognizes a band of the expected size of 40 kDa, only in the lane where the Ax2-Rad51 derived whole cell extract was loaded and not in Ax2-pDXA-3C derived extract (Figure 3.3C).

The generation of a strain overexpressing Rad51-2 allows us to perform subcellular fractionation experiments to determine whether Rad51 gets recruited to chromatin upon DSB induction. Rad51 cells were treated with phleomycin for various times up to 300 minutes and cell fractionation followed. Extracts prepared were analyzed by Western blotting using a monoclonal FLAG antibody. Rad51 is recruited to chromatin as early as 30 minutes upon damage induction and the signal peaks at 120 minutes and stays constant thereafter (Figure 3.4).

A


XbaI 7204
BamHI 7147
250...256 act15 terminator
FLAG-Rad51-2 6066...7147
Flag tag 6067...6090
KpnI 6061
HindIII 6044
1605...814 Tn5 neoR
act15 promoter 5700...6027
pDXA-3C-FLAG-Rad51 - 7214 nt
Ddp2 replication origin 5108...5684
AmpR 4128...3469

B


pDXA-3C
Rad51
XbaI
HindIII
8000bp
6000bp
1500bp
1000bp

C

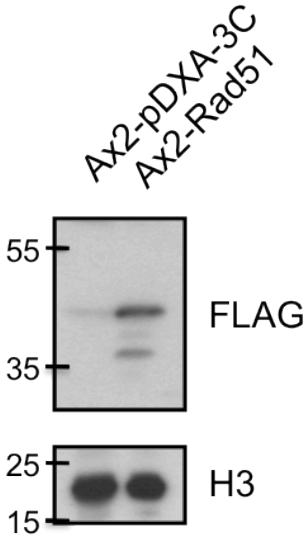

Ax2-pDXA-3C
Ax2-Rad51
55
35
FLAG
25
15
H3

Figure 3.3: Generation of the pDXA-3C-FLAG-Rad51 plasmid and strain verification of Ax2.Rad51 (A) Map of pDXA-3C-FLAG-Rad51 plasmid. The whole coding sequence of the Rad51-2 gene (DDB_G0273611) was amplified form cDNA with primers SL34 (bearing a FLAG tag and a KpnI restriction site) and SL33 (bearing a BamHI restriction site) (see Appendix A). The PCR product was purified and cloned into an intermediate vector by blunt end ligation. Subsequently, the product was cut out using the above restriction sites and cloned into the multi cloning site (MCS) of the pDXA-3C vector, which was digested with the same enzymes. Upon ligation the construct depicted above was generated. Sequence was verified by DNA sequencing. Promoter and terminator regions of the actin 15 gene are shown above, as well as the *Dictyostelium* origin of replication, Ddp2. AmpR, Tn5 NeoR: genes conferring resistance to ampicillin and neomycin and its analogues, respectively. (B) The pDXA-3C vector was digested with XbaI-HindIII, which cut adjacent to the beginning and end of the *rad51* coding sequence. The lower band observed corresponds to *rad51*, which is 1056bp long. (C) Whole cell extracts were prepared from both Ax2 cells, which served as a negative control and Ax2 cells expressing the pDXA-3C-FLAG-Rad51 plasmid. Equal amount of the extracts were subject to SDS PAGE electrophoresis and western blotting with an antibody raised against the FLAG epitope. Histone H3 serves as am indicator of equal loading of the two samples.

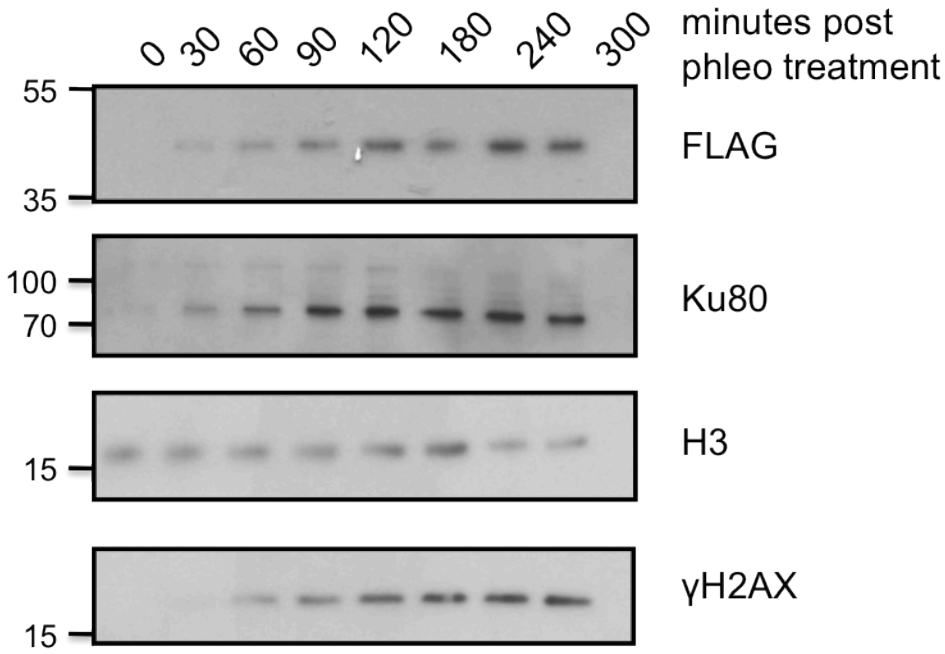


Figure 3.4: Rad51 gets recruited to chromatin in a time-dependent manner upon DSB induction, in a manner similar to Ku80. Exponentially growing Ax2-Rad51 cells were treated with phleomycin for various times up to 300 minutes. Cell fractionation followed. The experiment was performed as described previously (Section 3.3.2). The chromatin enriched extracts were analyzed by SDS PAGE electrophoresis using the relevant gel percentages for each protein analyzed (10% for Rad51 and Ku80, 15% for H3 and γH2AX). Western blotting followed with the antibodies indicated. The anti-FLAG antibody was used to probe for recombinant FLAG-tagged Rad51.

3.3 Discussion

Starting from a bioinformatic search, we attempted to identify proteins implicated in HR-mediated repair that correspond to the human counterparts. We were able to identify several proteins that are conserved between the two organisms (Table 3.1).

In previous studies, when NHEJ was addressed in *Dictyostelium* [109, 110], we were surprised to find that many proteins thought to exist only in higher eukaryotes, could also be found in *Dictyostelium.* The most prominent examples of this are the metallo-β-lactamase (MBL) (β- CASP-MBL) family nuclease *dclre1*, an ortholog of the vertebrate Artemis [110], along with DNA-dependent protein kinase catalytic subunit (DNA-PKcs) [109].

Given the above, I wanted to examine whether the same high degree of conservation existed between *Dictyostelium* and humans in HR proteins. The main body of proteins involved in HR repair in humans seems to be well conserved in *Dictyostelium,* as shown by identification of putative orthologs of proteins involved in all the different steps of the process (Table 3.1). Interestingly, *Dictyostelium* lacks Nbs1, a component of the MRN complex. The MRN complex recognizes DSBs and recruits ATM kinase, a PIKK kinase that has a central role in the DNA damage response [132]. Most interestingly, *Dictyostelium* does not appear to have an ortholog of ATM either. The above observations imply that *Dictyostelium* has evolved to respond to DNA damage utilizing different components of the DDR than other eukaryotes, in which ATM is conserved. This might involve the other two PIKK kinases that are conserved

in the organism, such as DNA-PK and ATR. However no further conclusions can be drawn in the present study. Another interesting observation is that *Dictyostelium* appears to have orthologs of all RAD51 paralogues identified in humans, when yeast only has Rad55 and Rad57, which seem to function in a similar way to the five human paralogues, but are not closely related to them [127]. Additionally, where *Dictyostelium* possesses orthologs of both BLM and WRN helicases, yeast only has one related helicase Sgs1 [142]. Finally, *Dictyostelium* seems to have an ortholog of BRCA2, when yeast does not. This is a very important finding, since BRCA2 has a central role in human HR, where it mediates steps essential for homology search and strand invasion, the most important steps for initiation of HR [61]. All the above suggest that *Dictyostelium*, could be a good model for study of HR in eukaryotes, due to the similarity the HR pathway shows to human HR.

Seeking to study the biochemistry of DNA repair, I adopted an assay previously described, for use in *Dictyostelium* and used it to monitor kinetics of recruitment of DNA repair proteins on damaged chromatin [124]. The method described in Section 3.3.1 is particularly useful in cases of proteins with high mobility and low abundance at the sites of breaks, such as Ku80 [140]. The results of our experiments (Figure 3.1 and 3.2) agree with those performed in other organisms, where Ku80 gets recruited to chromatin rapidly upon DSB induction to be followed by Rad51 [39, 138, 143]. Although in yeast Rad51 recruitment can only be observed in the S/G2 phase of the cell cycle, this is not the case in human cells where the protein gets recruited on damage sites in both G_1 and G_2/S phases of the cell cycle with similar kinetics [39, 138]. In vegetative *Dictyostelium* cells, the predominant pathway for repair is HR [110], as

illustrated by the finding that mutation of *ku80* or *dnapkcs* does not influence the ability of vegetative cells to repair DSBs [109, 114]. However, when the HR pathway is compromised by mutation of Exonuclease 1, the cells take a longer time to recover from damage administration [110]. The data showing chromatin recruitment of Rad51 upon damage administration in a time-dependent manner (Figure 3.4) agree with the above findings, showing that the HR pathway is indeed utilized upon DSB administration to vegatatively growing *Dictyostelium.*

At the same time, in other organisms Rad51 is recruited to chromatin significantly later than Ku80, which gets rapidly recruited to breaks. *Shibata et al.* have propose that when damage is administered in G_2, DNA ends are first bound by NHEJ protein [139]. However, if NHEJ fails to repair the break, HR next attempts to repair the break [139]. Growing *Dictyostelium,* at the vegetative, exponential phase of cell growth, have been shown to exist predominantly at the G2 phase of the cell cycle [114]. It has previously been suggested *Dictyostelium* cells might at first attempt of to repair the damage by NHEJ before proceeding to end resection and HR [110]. It would be interesting to define by quantitative and sensitive methods whether Rad51 gets recruited to chromatin later than Ku80, which would demonstrate a conserved mode of repairing DSBs.

It has also been reported, that during the developmental stage of cell growth and when *Dictyostelium* cells form spores, NHEJ is essential for DNA repair [109, 110]. It would be interesting to define whether Rad51 is differentially recruited during the cell cycle in *Dictyostelium*.

In summary, the above results suggest that the HR pathway appears to be conserved in *Dictyostelium* with representatives of most components identified in humans. Most importantly, a BRCA2 ortholog, which shows domain conservation, can be found in the genome. Moreover, the key HR component Rad51 is recruited to chromatin in a time-dependent manner, agreeing with the data from research in other organisms.

Chapter 4: The Auxin Inducible Degron system and its validation in *Dictyostelium.*

4.1 Introduction

Having identified a putative ortholog of BRCA2 in the *Dictyostelium* genome, I wished to characterize its function in this organism. Firstly, bioinformatic search was conducted to identify potential domain conservation between the *Dictyostelium* protein and BRCA2-related proteins from other organisms. The human BRCA2 protein carries a set of unique domains, which cannot be found on any other known human protein [77, 85, 91]. However, most of these domains are conserved in BRCA2-related proteins identified in other organisms [87, 88, 90]. A thorough bioinformatic search showed good domain conservation for the *Dictyostelium* BRCA2 ortholog. This result further encouraged the study of the BRCA2 ortholog in *Dictyostelium* cells.

Experiments conducted primarily in humans, but also other organisms, has shown that BRCA2 has conserved function in meiosis and DSB repair [74, 86-90]. Specifically, inactivation of BRCA2 in human cells causes genome instability and gross chromosomal rearrangements [73]. Accordingly, in mouse and *C.elegans* embryos inactivation of Brca2 causes early embryonic lethality, while causing chromosomal instability in somatic cells of the two organisms [70, 74, 88]. I sought to determine whether BRCA2 has a role in DSB repair in *Dictyostelium.* Consistent with the above, a first attempt to disrupt the putative BRCA2 ortholog via HR-mediated integration was unsuccessful. Hence, I hypothesized that the function of the BRCA2 related protein in *Dictyostelium* cells is essential for cell viability. This hypothesis is further supported by

previous findings, which report that disruption of HR-related genes is extremely challenging in *Dictyostelium* [109, 113].

To circumvent this problem, I decided to adopt a system to conditionally deplete the BRCA2 ortholog in *Dictyostelium*. Several methodologies for the conditional expression/depletion of proteins have been previously described in *Dictyostelium*, but their efficiency is questionable. RNA interference, like in other organisms, can be useful, but greatly depends on the efficiency of the hairpin generated for a satisfying degree of knock down. Although the system might offer a satisfying degree of knock down, the cells need to be cultured and express the hairpin for several hours before depletion of the protein of interest can be observed [144]. Another alternative, is the TET ON/OFF expression system, where proteins, either endogenous or expressed from an extrachromosomal vector, are under the control of a Tetracycline inducible/repressible promoter [145]. Again, cells need to be cultured with tetracycline for several hours before the expression of the desired gene is induced or repressed [145, 146]. Seeking to establish a technique that would deplete proteins more rapidly, I turned to the AID degron system [147]. The basis of the system's function is explained below.

In plants, the growth hormone auxin coordinates many developmental processes and regulates plant growth by regulating other proteins' abundance and gene expression levels [148, 149]. One example of its action is the induction of rapid degradation of the AUX/IAA family of transcriptional repressors. Degradation is achieved through a specific form of the Skp1-Cul1-F-box (SCF) ubiquitin ligase, the SCF^{TIR1}. The desired substrate specificity is

achieved when SCF forms a complex with the F-box transport inhibitor response 1 (TIR1) protein, an auxin receptor. In the presence of auxin SCF^{TIR1} interacts with AUX/IAA proteins to ubiquitinate them and target them for rapid degradation by the proteasome [150].

Nishimura et. al [147], took advantage of the fact that although the SCF complex, in addition to the degradation pathway in which it is involved, is conserved in eukaryotic non-plant cells, the auxin response is not. No orthologs of the IAA/AUX transcriptional repressors or the TIR1 mediator can be found in eukaryotes other than plants. In light of this, the Auxin-Inducible Degron (AID) system was exploited to conditionally deplete proteins in chicken, mouse, hamster, monkey and human cells. Once cell lines are engineered to express the TIR mediator, and the system is induced with auxin, any protein carrying an IAA transcriptional repressor sequence (henceforth called the AID tag), is rapidly ubiquitinated and targeted for degradation (Figure 4.1). Using molecular cloning techniques, the AID tag can be fused to any endogenous protein. Upon induction of the system with auxin, the degradation is rapid and reversible, allowing for study of proteins whose complete depletion is lethal to cells. This system offers certain advantages compared to other inducible systems that function at the DNA or protein level.

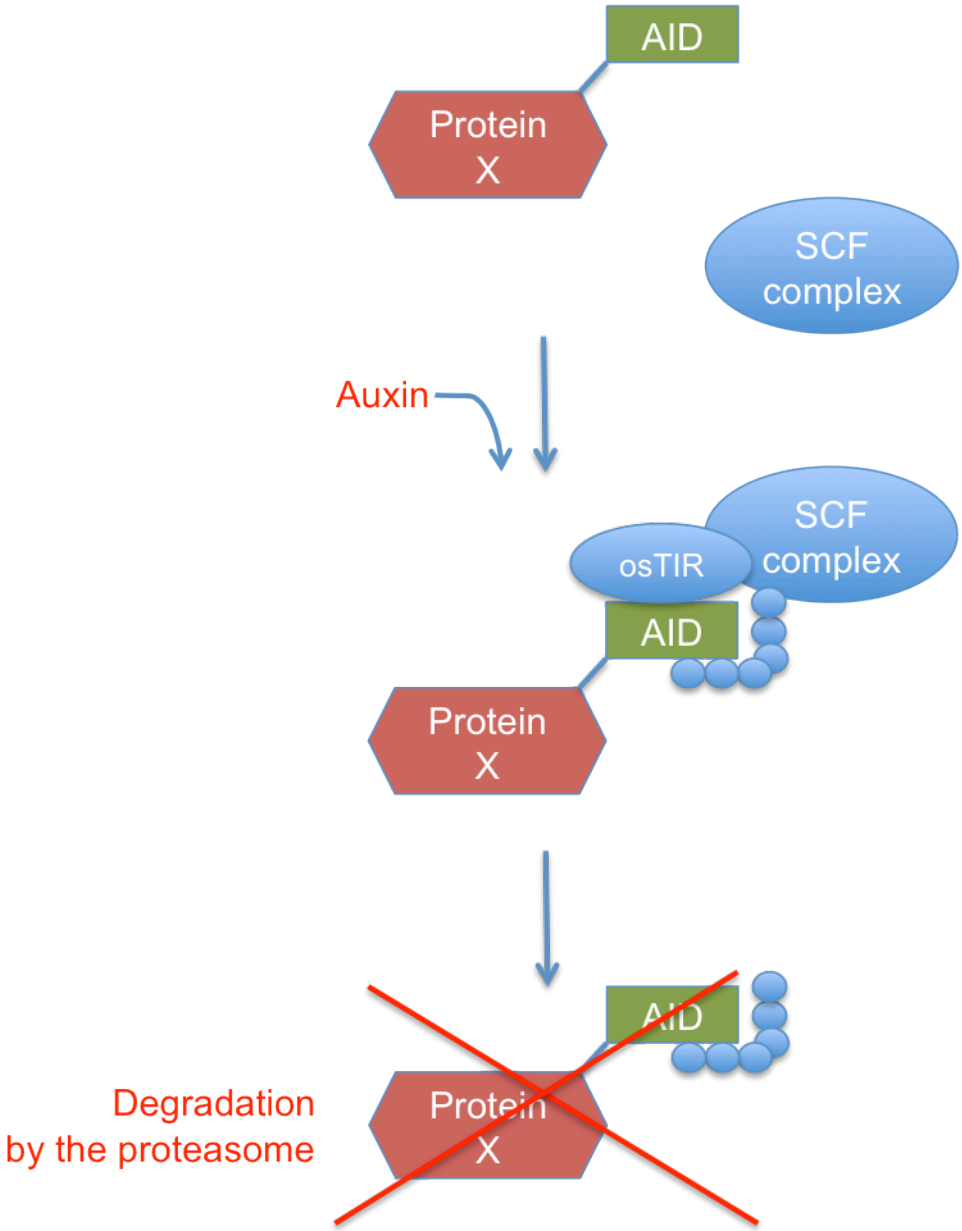


Figure 4.1: Schematic representation of the auxin degron system. The protein of interested is tagged with the AID tag. Subsequently, the cells, already expressing the osTIR mediator, are treated with auxin. Auxin interacts with osTIR and the SCF complex, which then specifically recognizes the AID-tagged protein and polyubiquitinates it targeting it for degradation.

All the above prompted us to test whether the system was applicable to *Dictyostelium* to ultimately use it to temporarily deplete the putative ortholog of BRCA2 from cells. Establishing this system will serve as a valuable tool for this project, as well as future work for the *Dictyostelium* community. What prompted me to establish the technique, was the fact that many DNA repair proteins, and more specifically HR proteins, prove to be essential for cell viability. In many cases, that has prevented us and other laboratories from characterizing these genes and their function [109, 113, 151]. In light of the above, I proceeded to establish the AID system to conditionally deplete the BRCA2-related protein from *Dictyostelium* cells so I can ultimately study its function. For the verification of the system, I initially established that the SCF complex is conserved. Subsequently, I tested the functionality of the AID system in *Dictyostelium* as described below.

4.2 Results

4.2.1 Certain key domains of BRCA2 are conserved in *Dictyostelium*

BRCA2 is a protein involved in HR where it functions to load Rad51 at resected DSBs [61, 75]. Importantly, loss of BRCA2 function results in early embryonic lethality [70]. Research conducted in vertebrate cells with hypomorphic BRCA2 mutants, shows severe genome instability in the form of gross chromosomal rearrangements, denoting the essential role of the protein for genome integrity and prevention of carcinogenesis [73, 75, 152]. Interestingly, no orthologs of BRCA2 can be found in lower eukaryotes such as yeast, although its function seems to be essential in mammals.

Human BRCA2 is a 3,418 amino acids long protein and consists of many conserved domains unique to BRCA2. Of those, the BRC repeats function in facilitating interaction of the with Rad51 [77-79]. The C-terminal 800 amino ssDNA Binding Domain (DBD) of the protein also interacts with Rad51 [71]. Its structure has recently been resolved, showing three types of oligonucleotide/oligosaccharide binding folds (OB folds): OB1 (cd:04493), OB2 (cd:04494) and OB3 (cd:04495), all of which interact with ssDNA. Preceding these, a Helical Domain (pfam09169) can be found which forms a structure with the OB1 fold that interacts with the 70 amino acids long acidic protein DSS1 [85].

BRCA2-related proteins have been identified and studied in various organisms [86-88, 90]. The identification of BRCA2 related genes in *U.maydis,* a fungus that infects corn, and the plant *A.thaliana* were the first indications that BRCA2's function as a mediator of Rad51 is conserved in lower eukaryotes as well as plants [87, 89]. All BRCA2-related proteins identified are nuclear proteins containing BRC repeats and at least one OB1 fold. Their size varies from one organism to another with the human protein being the largest (Figure 4.2). The putative ortholog of BRCA2 in *Dictyostelium* (DDB0306020) is a 1,616 amino acids long protein, containing three BRC repeats, which show 98% identity and 100% similarity to each other (Appendix C, Figure S2), along with a Nuclear Localization Signal (NLS) at its N-terminal portion. In addition, two copies of the OB1 fold preceded by a BRCA2 helical domain can be found at the C-terminus (figure 4.2). Hereafter the protein will be referred to as Brcp.

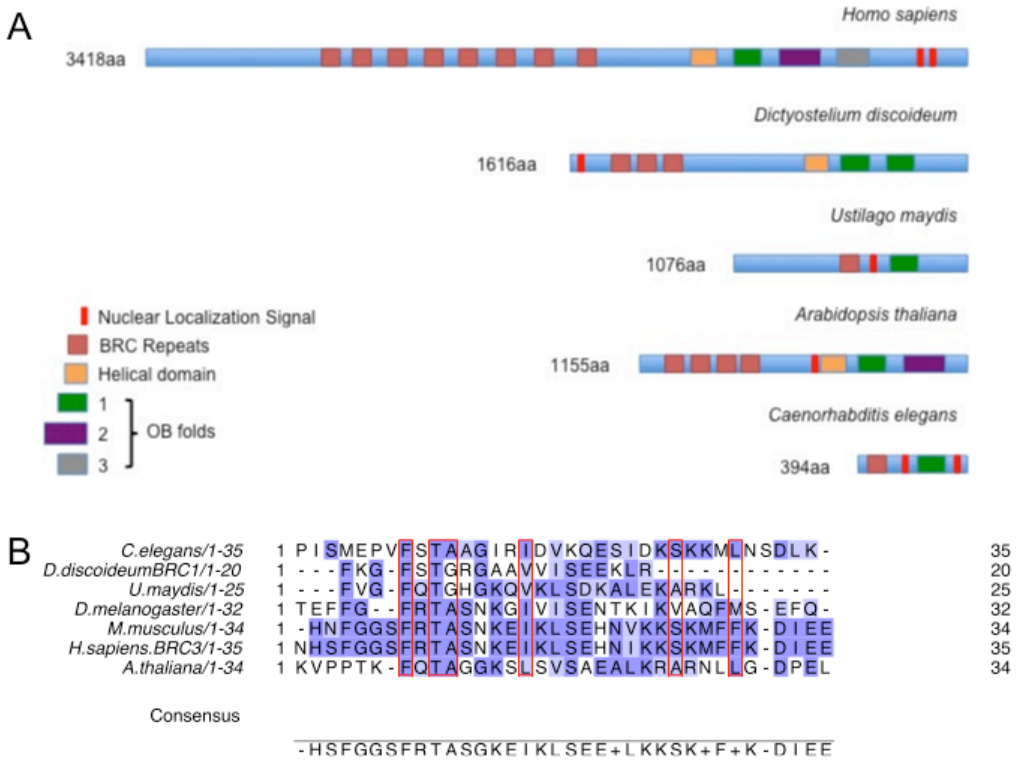


Figure 4.2: Key domains of BRCA2 conserved in the *Dictyostelium* putative ortholog. (A) BRCA2 like proteins and domain conservation across species**.** Domain architecture of the BRCA2 and BRCA2-like proteins that have been identified in species other than humans are shown. Nuclear localization Signal (red), BRC repeats (pink), Helical domain (ochra), OB1 (green), OB2 (purple), OB3 (grey) fold are shown. Sizes of the proteins and species of origin are indicated on the left and above each cartoon respectively. (B) The first *Dictyostelium* BRC repeat shows conservation of Rad51-interacting amino acids. Alignment of BRC repeats from different organisms. Dark blue coloring shows identical amino acids, whereas light blue similar ones. The red borders denote amino acids that are important for the interaction of human BRCA2 with Rad51, as defined by structural studies [91].

These findings suggest that DDB0306020 is a BRCA2 ortholog in *Dictyostelium.* To further establish this finding, I aligned the sequence of the first of the three BRC repeats in *Dictyostelium* with that of other known BRCA2-related proteins as well as the third repeat from human BRCA2 (Figure 4.2B). From the alignment, it becomes apparent that although the *Dictyostelium* sequence is significantly shorter than a typical BRC repeat, most of the amino acids identified to be important for interaction with Rad51 in the human protein are conserved (Figure 4.3, amino acids marked in red) [91]. BRC repeats are found in different numbers in different organisms in the identified BRCA2-related proteins. The human protein has 8 BRC repeats [77] and other mammals have similar numbers [78]. In the case of *C.elegans* only one repeat is present [88]. Intermediate numbers can be found in the plant *A.thaliana,* the fly *D.melanogaster* and the fungus *U.maydis* orthologs [86, 87, 89].

All the above suggest that the repeats identified in Brcp show some conservation when aligned to BRC repeats found in other organisms. It is important to note that three of the residues that have been shown to be important for interaction with Rad51 are conserved. This finding reinforces the hypothesis that Brcp might be an ortholog of BRCA2 that acts as a mediator of *rad51* in *Dictyostelium.*

When the above sequences are used to construct a phylogenetic tree, the *C.elegans* repeats serves as an outgroup, having diverged the most. *U.maydis* and *Dictyostelium* belong to the same clade, which have diverged from the fruit fly and mammals after their common ancestor diverged from plants (represented by *A.thaliana*) (Figure 4.2C).

4.2.2 BRCA2 function appears to be essential for cell survival in *Dictyostelium discoideum.*

Having identified the putative ortholog of BRCA2 in *Dictyostelium* (see section 3.2.2), I sought to determine its biological function. I cloned a fragment of 543bp and a second of 690bp corresponding to the 5' and 3' end of the *brcp* gene (nucleotides 1-543 and 4157-4851 of the coding sequence) into the pLPBLP disruption vector [123]. The two regions flanked a blasticidin resistance cassette (Figure 4.3, top panel). Upon linearization, the targeting construct was electroporated in Ax2 cells. Cells were left to recover for one day and subsequently selected in blasticidin for 10 days.

In the event of successful integration, the targeting construct and genomic sequence will undergo homologous recombination, which will result in disruption of gene sequence and function. Clones were selected with blasticidin. Surviving clones were screened by PCR with primers annealing outside the region of the integration (SL16-SL17, Figure 4.3). These primers, in the case of successful integration would amplify a product significantly smaller than the full-length *brcp* due to the substitution of the gene by the disruption construct. However, after screening 300 colonies, I was unable to identify any targeted integration events. This is possibly due to the essential role of Brcp in *Dictyostelium.* This argument is supported by the attempts of another group to disrupt the *brcp* gene that were equally unsuccessful [113].

In light of the above, I decided to establish a conditional protein depletion system that is based on the AID system described above.

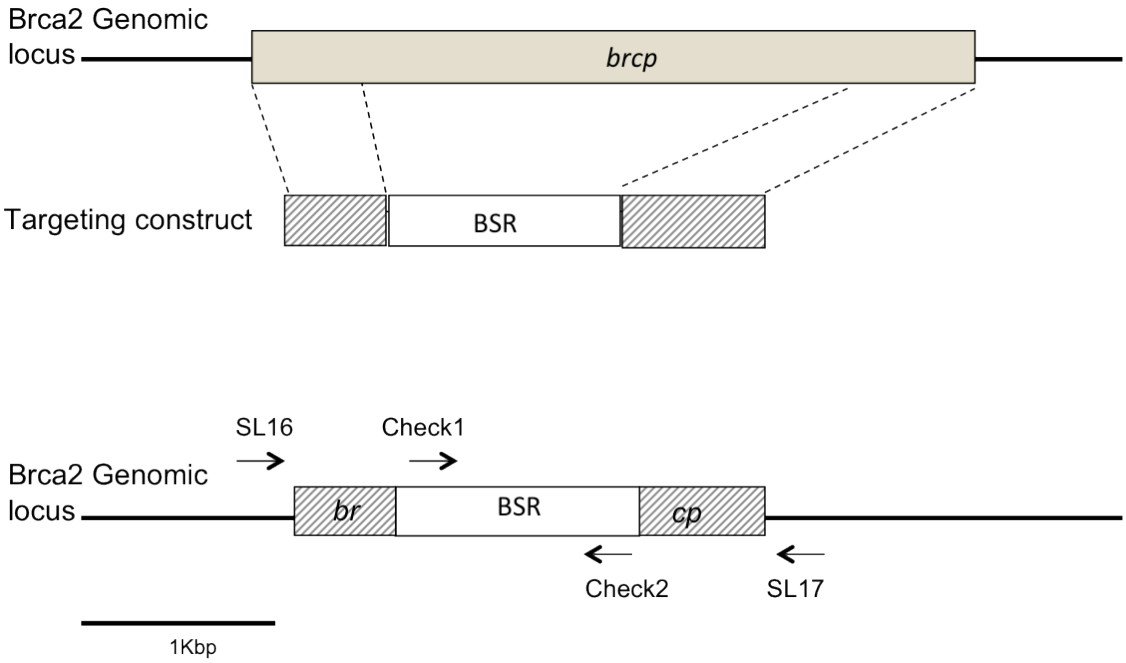


Figure 4.3: Disruption of the *brcp* gene. Regions of 543bp (using primers SL11-SL12) and of 690bp (using primers SL09-SL10) at the beginning and end of the gene, respectively were amplified by PCR and cloned in the pLPBLP plasmid [123], upstream and downstream of the blasticidin resistance cassette, respectively. Successful integration of the arms was verified by restriction digestion and sequence by DNA sequencing. The construct was amplified and digested with KpnI and BamHI to result in the production of the targeting construct. Upon digestion, DNA was purified with phenol-chloroform extraction and 8µg electroporated in Ax2 cells. Selection with blasticidin (10µg/ml) followed and after two weeks colonies were either pooled together or individually screened for successful integration using the indicated primers. SL16 and SL17 were used for screening for a targeted integration events, while Check1- Check2 were used as a positive control reaction (data not shown).

4.2.2 The components of the SCF complex are conserved in *Dictyostelium* while the AUX/IAA sequences cannot be found.

One key issue that we had to address before setting out to establish the auxin system was whether the SCF ubiquitin ligase complex is conserved in *Dictyostelium*. When I queried dictyBase (www.dictybase.org) for orthologs of the components of SCF, I found that all of them (orthologues of SKP1, CUL1 and RBX1) are represented and expressed in vegetative cells (data not shown). Moreover, the SCF complex and its function have been studied and are conserved in *Dictyostelium* [153].

To test whether an auxin response exists in *Dictyostelium*, cells were grown on a *Ka* lawn on solid medium plates containing auxin. Importantly, continuous exposure to auxin did not have an effect on *Dictyostelium* growth and development, since colonies appeared at the expected times and fruiting bodies formed normally (data not shown). Also, when the sequence of the IAA transcriptional repressor (IAA17 or ARX3 in *A.thaliana* A.N. NM_100306), which is used as the AID tag, was aligned against the *Dictyostelium* genome, no similar sequence were recovered, indicating that this aspect of the auxin response is most likely absent from this organism. This finding shows that the possibility of an auxin response existing in *Dictyostelium* is low.

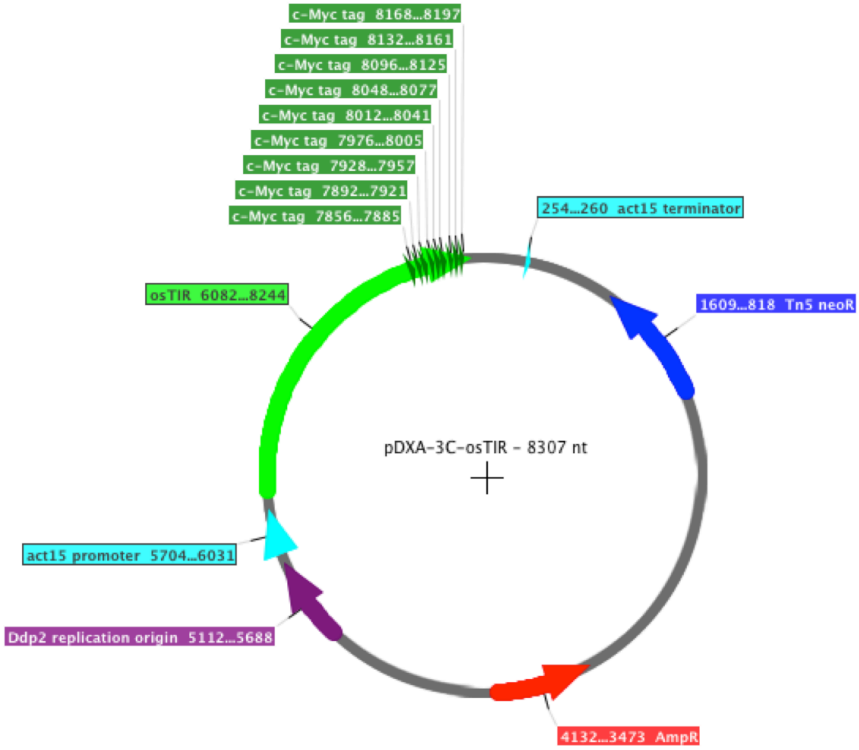
All the above, indicate that all the basic components needed for the establishment of the AID system are present and also an auxin response is absent in *Dictyostelium* cells. Hence, it is possible that the TIR mediator could interact with the orthologs of the SCF complex found in *Dictyostelium.* To

subsequently test whether the AID degron system could function in *Dictyostelium*, I created a cell line that expressed osTIR along with AID-tagged GFP. The cell line was used to test whether treatment of the osTIR expressing cells with auxin would trigger degradation of GFP-AID.

4.2.3 Establishment of cell lines to test the AID system in *Dictyostelium.*

To establish the AID degron system in *Dictyostelium,* I first wanted to examine whether a test protein tagged with AID is degraded in response to treatment with auxin in cells constitutively expressing TIR1. TIR1 originating from *Orisa sativa* (rice) (original plasmid provided by C. Eichinger/ Nasmyth laboratory) was cloned in pDXA-3C, which contains a G418 resistance cassette (A.N. X85118) [141]. The rice TIR1 protein is more stable than the *A.thaliana* protein. The cloned fragment was excised from an original plasmid, which contained osTIR, along with nine myc tags, so that the protein expression can be easily verified by western blotting (Figure 4.4A). The Green Fluorescent Protein (GFP) tagged with AID and a nuclear localization signal (NLS), was also cloned in another expression vector pDM326, which contains a Blasticidin resistance cassette (GeneBank EU912541.1) [154] (original plasmid for both constructs from Dr. C.Eichenger and Prof.K. Nasmyth, unpublished material).

A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c-Myc tag 8012...8041
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c-Myc tag 7928...7957
c-Myc tag 7892...7921
c-Myc tag 7856...7885
254...260 act15 terminator
1609...818 Tn5 neoR
osTIR 6082...8244
pDXA-3C-osTIR - 8307 nt
act15 promoter 5704...6031
Ddp2 replication origin 5112...5688
4132...3473 AmpR

B

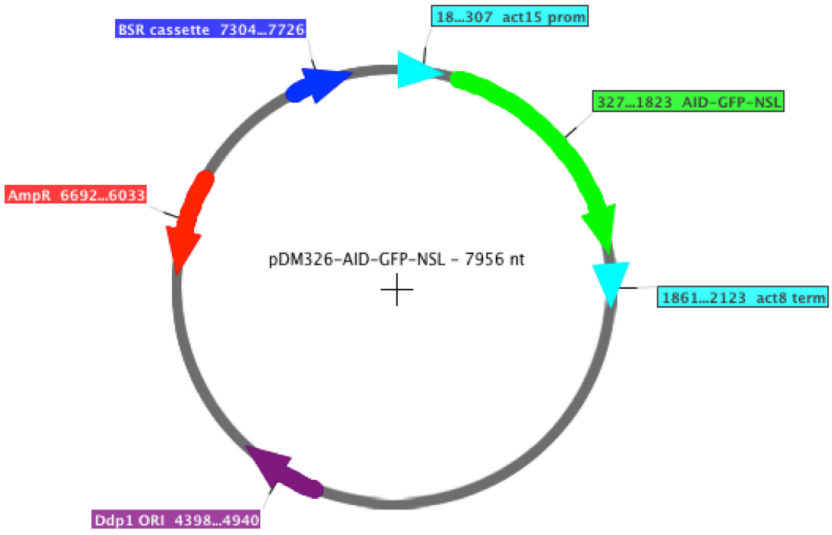
BSR cassette 7304...7726
18...307 act15 prom
327...1823 AID-GFP-NSL
AmpR 6692...6033
pDM326-AID-GFP-NSL - 7956 nt
1861...2123 act8 term
Ddp1 ORI 4398...4940

Figure 4.4: Cloning of osTIR and AID-GFP-NLS in Dictyostelium expression vectors. (A) osTIR-9xmyc was excised from the parental plasmid by restriction digestion, using AfeI and SmaI. Subsequently, pDXA-3C was digested with BamHI and the overhangs generated were filled in using the Klenow fragment of DNA polymerase I. Blunt end ligation followed. The resulting construct is displayed above, containing a G418 (Tn5/neoR) and Ampicillin(AmpR) resistance cassette. Actin 15 promoter and terminator are shown and drive the expression of the cloned gene (osTIR-9xmyc). An origin of replication for *Dictyostelium* (Ddp2) is also shown. (B) The GFP-AID-NLS was amplified by PCR from the parental plasmid using primers SL25 and SL26 (Appendix A). Sequence was verified by DNA sequencing and the fragment was cloned into pDM326. The plasmid was digested with BglII and overhangs were filled as in (A). Blunt end ligation followed. Promoter and terminator elements that drive the expression of the transgene are shown in cyan, blasticidin resistance cassette in blue. The ampicillin resistance gene is shown in red and the *Dictyostelium* replication origin in purple.

Cells were transfected with both pDXA-3C-osTIR and pDM326-GFP-AID-NLS and grown on 90mm petri dishes under blasticidin and G418 selection. Control strains were created containing pDM326-GFP-AID-NLS and empty pDXA-3C or empty pDXA-3C and pDM326 plasmids. Both nuclear and whole cell extracts were prepared from all strains and analyzed by Western blotting, with the indicated antibodies, to validate expression of the recombinant proteins. I also wanted to test whether a higher concentration of G418, used for selection of the pDXA-3C plasmid, would have an impact on osTIR expression levels. A GFP signal can be detected in all GFP-AID transfected cell lines, although expression levels in the strain transfected with pDM326-GFP-AID-NLS and empty pDXA-3C is low. GFP levels are also lower in the case for the strain expressing AID-GFP and osTIR being selected with 40μg/ml of G418, when compared to the strain selected with 20μg/ml. osTIR levels remain unaffected by higher concentration of selection (Figure 4.5). For this reason, 20μg/ml of G418 were used in all subsequent experiments.

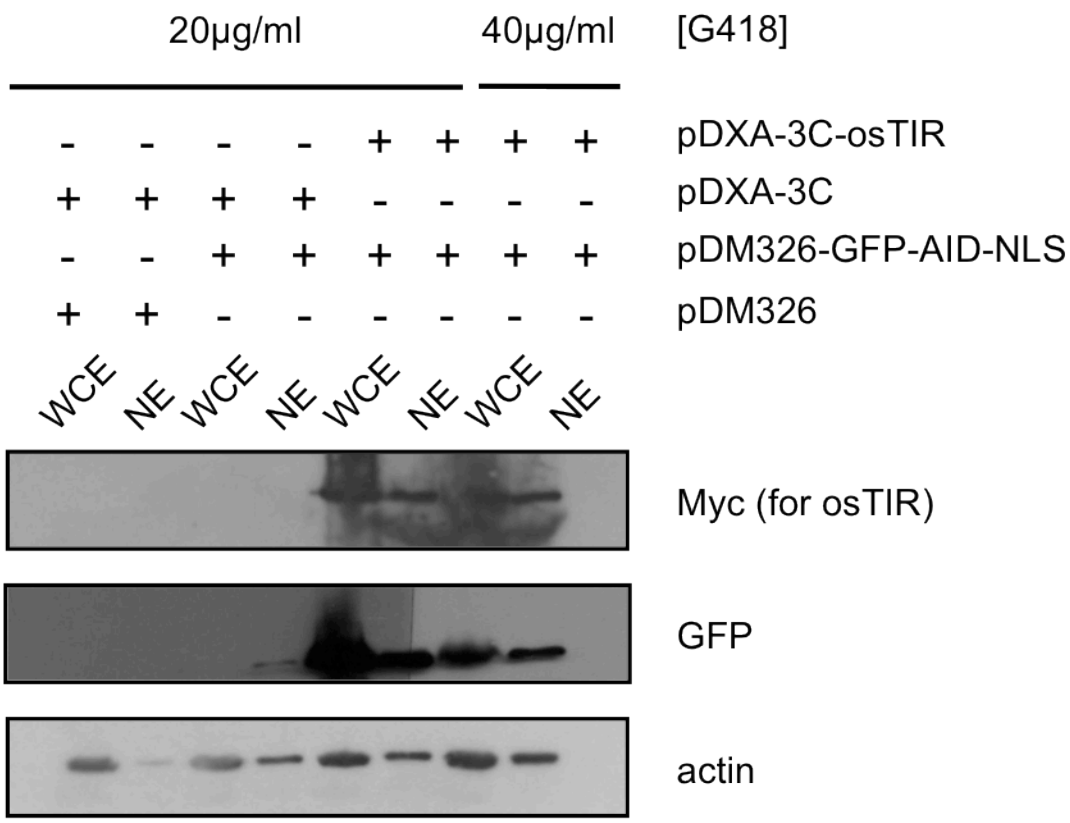

Figure 4.5: osTIR and GFP-AID-NLS can both be detected after transformation of Ax2 cells with the expression vectors carrying the transgenes. Nuclear (NE) and whole cell (WCE) extracts were prepared by nuclear extraction or by resuspending in SDS loading buffer and boiling, respectively, 1 x 10^7 cells. Of that, an equivalent of 2 x 10^6 cells was loaded and analysed by western blotting. Antibodies used for the experiment are shown, as well as plasmids with which each cell line has been transformed. Finally, the concentration of G418 is displayed at the top.

4.2.4 Titration of the auxin response

Having established strains that express osTIR and GFP-AID, I wanted to test whether treatment of these cells with auxin would result in degradation of AID-tagged GFP. This response has been previously observed when tested in cell lines derived from various organisms [147]. The strain generated, expressing both pDXA-3C-osTIR and pDM326-GFP-AID-NLS, as well as the control strains expressing empty pDXA-3C and pDM326-GFP-AID-NLS or empty pDXA-3C and pDM326, were treated with different concentrations of auxin for two hours. The experiment shows that in cells expressing both osTIR and GFP-AID-NSL, treatment with increasing concentrations of auxin for two hours results in marked reduction in the levels of AID-GFP when compared to mock treated cells. However, when cells expressing only GFP-AID-NSL are treated with the same amounts for the same period, no reduction in the GFP levels detected by western blotting can be observed. This observation indicates that the GFP is degraded in an auxin and osTIR-dependent manner (Figure 4.6A).

Having established the osTIR-dependency of the auxin response, I wanted to titrate the optimal time and dose needed for maximal degradation. I treated cells with 0.25mM, 0.5mM and 1mM of auxin and for increasing periods of time up to 120 minutes. When cells are treated with 0.5mM of auxin, the GFP signal decreases over time more rapidly than when 0.25mM is used (Figure 4.6B). However, when cells were treated cells with 1mM of auxin, the kinetics of GFP degradation seem to be the same as in the 0.5mM. Accordingly, 0.5mM is the concentration of auxin that will be used for all subsequent experiments.

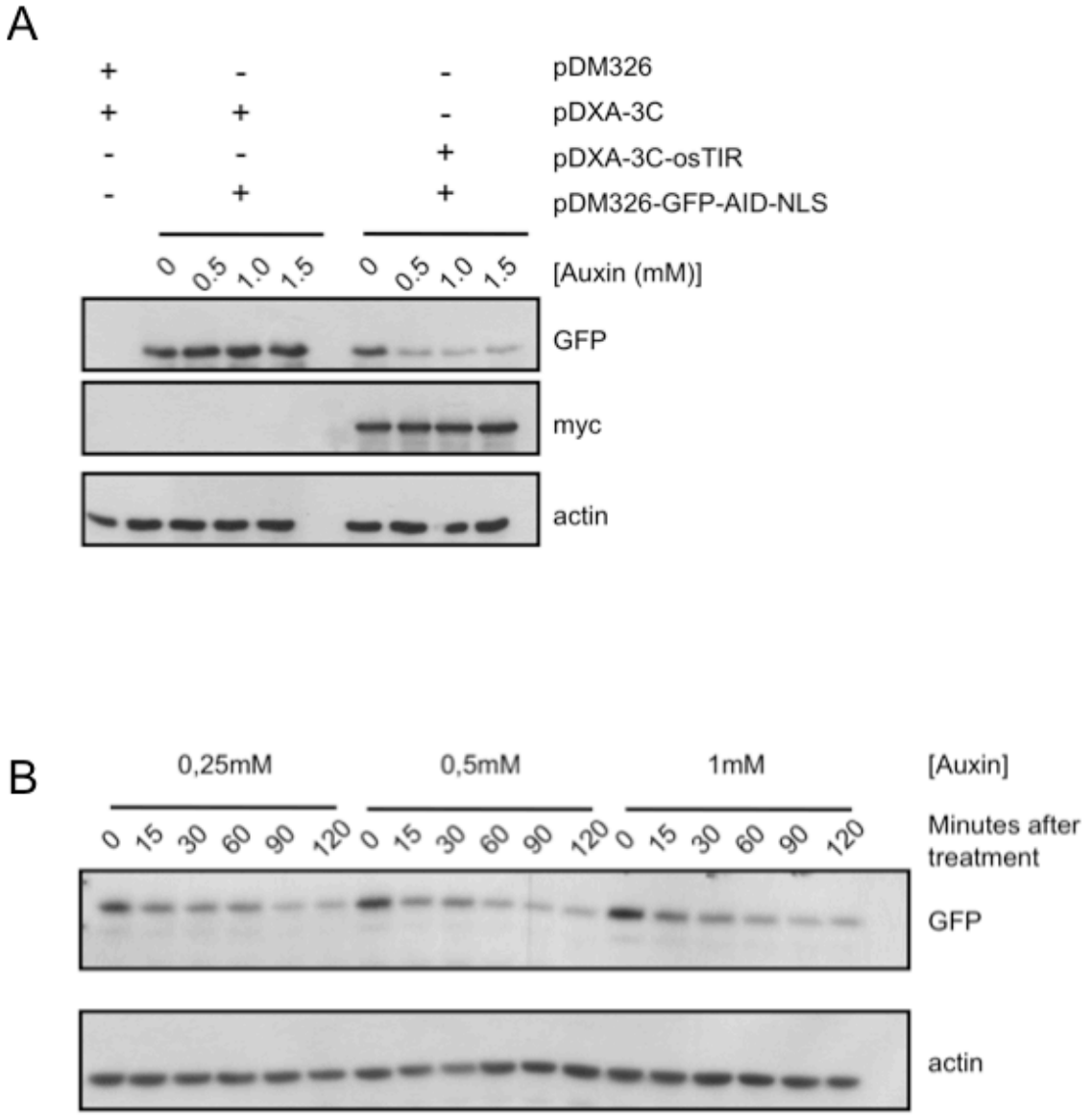

Figure 4.6: Titration of the auxin response (A) GFP gets degraded upon auxin treatment in an osTIR-dependent manner. The indicated cell lines were treated with the above indicated increasing concentrations of auxin for two hours. Following treatment, whole cell extracts were prepared by resuspending 2 x 10^6 cells in SDS loading buffer and boiling them. Equivalent amounts of all extracts were analysed by western blotting with the indicated antibodies. (B) Treatment with auxin causes degradation of GFP over time. Cells were treated with the indicated concentrations of auxin for the indicated times. Whole cell extracts were prepared from 2 x 10^6 cells by resuspending in SDS loading buffer and boiling. Same cell number equivalents were analysed by western blotting with the indicated antibodies.

4.3 Discussion

Through an *in silico* search, I managed to identify a putative ortholog of BRCA2, in *Dictyostelium* (Brcp). BRCA2 is an essential mediator of Rad51 in human HR [61] but has no orthologs in organisms such as yeast. When BRCA2 is mutated in human, mouse and worm cells, genomic instability is induced in somatic cells [70, 74, 88]. Additionally, inactivation of the gene causes early embryonic lethality in mice and *C.elegans* [70, 88]. The domain structure of the human protein has been well defined [77, 85, 91]. Interestingly, BRC repeats, as well as the C-terminal portion of BRCA2 show different degree of conservation in BRCA2-related identified other organisms such as mice, chicken, worm, fungi and plants [78, 87-90]. In *Dictyostelium*, we find three of the internal repeats found on BRCA2, the BRC repeats, to be conserved. Although the repeats are smaller than those of all other characterized orthologs, a few of the key amino acids required for interaction with Rad51 in humans, defined by structural studies, are conserved (Figure 4.2) [91]. The fact that only three repeats are present in the *Dictyostelium* ortholog when the human counterpart possesses 8 is not surprising. The *C.elegans* BRCA2 ortholog only possess one repeat [88] and all other characterized orthologs have varying numbers of BRC repeats (from 1-7) [78, 87, 89, 90]. Apart from the BRC repeats, two other BRCA2 conserved domains, the BRCA2 helical domain and an OB1 fold, are in close proximity on Brcp. The same two domains have been found to form a complex three-dimensional structure binding the small acidic protein DSS1 and interacting with ssDNA on the human counterpart [85]. All the above indicate that Brcp is highly likely to be a BRCA2 ortholog and encouraged me to further investigate the cellular function of Brcp in *Dictyostelium*.

To this end, I initially attempted to disrupt the gene in *Dictyostelium* cells (Figure 4.3). However, these attempts were not successful. We and others have found disruption of HR genes in *Dictyostelium* to be challenging [109, 113, 151]. It is very likely that in *Dictyostelium,* a haploid organism, disrupting the only copy of a gene that contributes to the repair of the most toxic form of DNA lesions can be detrimental for cell viability. Favoring the idea that Brcp might be essential for cell viability, I sought to develop a conditional depletion system. The lack of efficient conditional systems in *Dictyostelium* prompted me to develop a novel system for conditional protein depletion. This system was selected mainly because of its rapid and reversible induction [145, 155]. With a view to ultimately apply this system to Brcp, the functionality of the system in *Dictyostelium* was established by degrading a test protein (AID-GFP-NLS) and showing I can the protein in response to auxin treatment (Figure 4.6).

AID-tagged GFP was degraded in an osTIR- and auxin-dependent manner in *Dictyostelium* cells as previously displayed in yeast, human, monkey, hamster and mouse derived cell lines [147]. The fact that GFP signal can be detected even after two hours of treatment with auxin (Figures 4.5 and 4.6) is attributed to the fact that the protein is being over expressed. Instead, in the original experiment, conducted in yeast cells, only one copy of AID-GFP was integrated in the genome [147]. Hence, the levels of expression were significantly lower and the stoichiometry of production versus depletion different, potentially favoring a more efficient degradation. The above finding suggests that the AID degron system can be used in *Dictyostelium* to conditionally deplete cells of

desired proteins, providing researchers working with this organism with a novel conditional protein depletion system.

Chapter 5 : Phenotypic study of a Brcp-AID strain

5.1. Introduction

BRCA2 is a protein essential for HR in humans and other organisms, where its inactivation causes genome instability and GCR [73-75, 86-90]. Additionally, inactivation of the protein results in early embryonic lethality in mouse and *C.elegans* embryos [70, 88]. Having identified an putative ortholog of BRCA2 in *Dictyostelium*, we sought to study its function and potential involvement in HR in this organism. An initial attempt to disrupt the gene was unsuccessful, suggesting that Brcp function might be essential for cell viability. This hypothesis is supported by the findings of another group, which was also unable to disrupt *brcp* [113]. In order to circumvent this problem, I established that the AID degron system (see chapter 4) can be used in *Dictyostelium* to conditionally deplete AID-tagged proteins. To extend these findings, I wanted to verify that the AID degron system can be used to deplete endogenous protein, in this case Brcp from cells. To this end, I created a *Dictyostelium* strain containing a version of Brcp fused to 3 Hemagglutinin (HA) and the AID tag. In this strain, when osTIR was expressed and cells treated with auxin, rapid degradation of Brcp is observed. The above finding illustrates that the system can be used for conditional depletion of endogenous proteins in *Dictyostelium*. The generated strain was then utilized to study the role of Brcp in *Dictyostelium* cells, mainly to assess a potential role for Brcp in DSB repair by HR.

Interestingly, a recent observation in human cells suggests a synthetically lethal relationship exists between BRCA2 and PARP1 inactivation [98]. The basis of

this relationship has been implied but is not clear [98]. Based on the above findings, PARP inhibitors are being tested as an anticancer drug. As cancer cells are highly adaptive, there have been reports showing that human cells, deficient in either BRCA1 or BRCA2, can develop resistance to PARP inhibitors after treatment, either through reversion mutations of BRCA2, or through inactivation of other genes implicated in the DDR [119, 120, 156]. Verification of Brcp as an ortholog of BRCA2 would give us the opportunity to explore a potential synthetic lethal relationship with PARPs, which are conserved in *Dictyostelium* with certain members of the family implicated in DNA repair [112]. In an organism as simple as *Dictyostelium*, molecular dissection of this synthetic lethal relationship and the bases of resistance to PARPi could be examined, through generation of random mutant clones and screening for resistance to PARP inhibitors.

5.2 Results

5.2.1 Generation of a cell line containing an AID-tagged version of the endogenous Brcp.

To target Brcp for degradation using the AID degron system, it was necessary for the endogenous gene to be tagged with AID (see chapter 4). Additionally, to facilitate detection of the protein in cell extracts, three copies of the hemagglutinin (HA) epitope tag were introduced adjacent to the AID tag at the C-terminus of the protein.

Several steps were needed to generate a strain containing an AID and HA-tagged version of Brcp. Initially, I attempted to amplify the full-length *brcp* gene through PCR. However, *Dictyostelium* is an organism whose genome is very A-

T rich [103], making amplification of large genomic sequences challenging. In order to circumvent this problem, I amplified four pieces of the *brcp* gene, which I then fused together using restriction sites endogenous to the genomic sequence (Figure 5.1Aa). A fragment corresponding to nucleotides 3801-4847 (1051bp long, the stop codon of *brcp* is not included) was also amplified to contain an engineered SalI site at the 3' end and used to construct a 3xHA and AID-tagged version of the Brcp C-terminus (Figure 5.1Ac). 3xHA-AID was cloned adjacent to the *brcp* fragment, using the endogenous SalI and SmaI sites. Finally, 70bp oligonucleotide important for termination of transcription [157] was fused 3' to the 3xHA-AID using engineered restriction sites SmaI and PstI. Upon restriction digestion with the relevant enzymes (described in Figure 5.1A), fragments from the aforementioned constructs were used to generate a large segment of the *brcp* gene (4,676bp) followed by the sequence coding for 3xHA and the AID tag, the "5' arm", which was then inserted in the integration vector pLPBLP (Figure5.1A and B). For the generation of the "3' arm" of the construct, a sequence of 1460bp corresponding to the sequence immediately downstream of *brcp* was cloned in an intermediate vector (Figure 5.1A and B). The cloning process is described in detail in Figure 5.1A.

In summary, a construct was engineered to contain the "5' arm", a large segment of the *brcp* followed by 3xHA and an AID tag, followed by LoxP flanked blasticidin resistance cassette. 3' to the BsR cassette, the "3' arm" was inserted (Figure 5.1B). Purified plasmid was then digested with PmlI and PshAI, sites endogenous to the genomic sequence and purified. 8µg of the purified DNA were transfected in *Dictyostelium* Ax2 cells by electroporation.

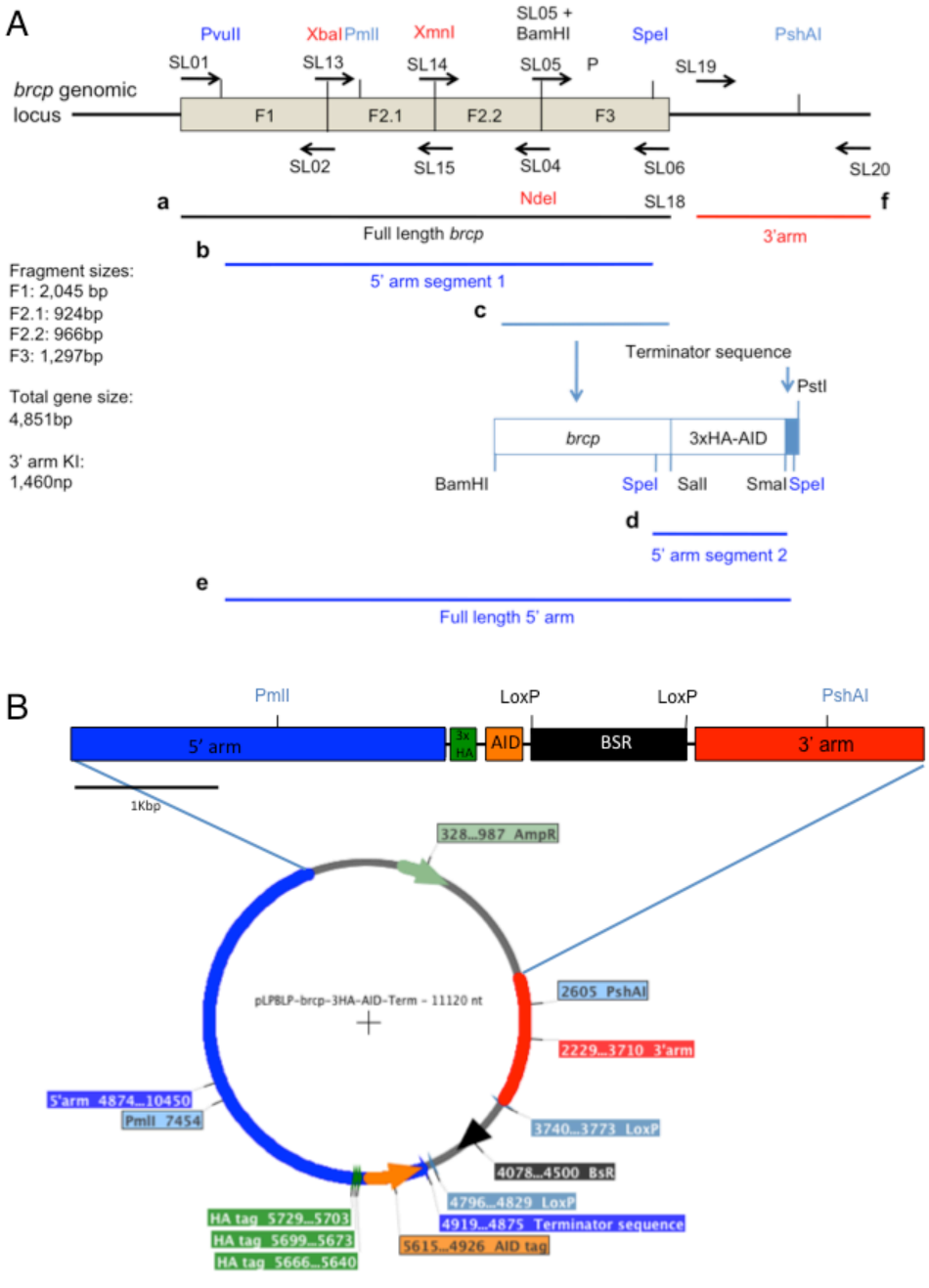
A
PvuII
XbaI PmlI
XmnI
SL05 +
BamHI
SpeI
PshAI
SL01
SL13
SL14
SL05
P
SL19
brcp genomic
locus
F1
F2.1
F2.2
F3
SL02
SL15
SL04
SL06
SL20
a
NdeI
SL18
f
Full length brcp
3'arm
b
5' arm segment 1
c
Terminator sequence
PstI
brcp
3xHA-AID
BamHI
SpeI
SalI
SmaI
SpeI
d
5' arm segment 2
e
Full length 5' arm
Fragment sizes:
F1: 2,045 bp
F2.1: 924bp
F2.2: 966bp
F3: 1,297bp
Total gene size:
4,851bp
3' arm KI:
1,460np
B
PmlI
LoxP
LoxP
PshAI
5' arm
3x HA
AID
BSR
3' arm
1Kbp
328...987 AmpR
pLPBLP-brcp-3HA-AID-Term - 11120 nt
2605 PshAI
2229...3710 3'arm
5'arm 4874...10450
PmlI 7454
3740...3773 LoxP
4078...4500 BsR
4796...4829 LoxP
4919...4875 Terminator sequence
HA tag 5729...5703
HA tag 5699...5673
HA tag 5666...5640
5615...4926 AID tag

Figure 5.1: Creation of pLPBLP-*brcp*-3xHA-AID-term construct. (A) The *brcp* genomic locus is depicted. Four fragments were amplified by PCR (F1, F2.1, F2.2, F3) using the indicated primers. All fragments amplified overlap with each other at the first ~20bp. Endogenous restriction sites within the overlapping sequences, indicated in red, were used for the fragments to be pasted together. Primers SL01 and SL06 harbour engineered BamHI sites, which were used for cloning the full length gene (a) in the pDM309 vector (full map not shown). From digesting (a) with PvuII and SpeI, the (b) fragment was generated. At the same time, F3 was amplified using SL05 containing a BamHI site and SL18 containing a SalI site. The fragment was then cloned in the intermediate vector PSP72 (A.N. X65332.2). SL18 has been designed so that the *brcp* fragment amplified does not contain the endogenous stop codon of *brcp.* Three HA tags and the AID tag were cloned, in frame with the *brcp* fragment, using endogenous SalI and SmaI sites (isolated from the original pmk4-3HA plasmid provided by C. Chan/ Kim Nasmyth's laboratory). The AID gene contains a stop codon. A small terminator sequence important for termination of transcription [157] was cloned following the 3xHA-AID. I generated this small dsDNA sequence by annealing complementary ssDNA oligonucleotide *in vitro* and digesting them with restriction enzymes recognizing sites I had engineered upstream and downstream to the terminator sequence, SmaI and PstI, respectively. The oligonucleotide was then ligated to the rest of the construct. A nested SpeI engineered following the terminator sequence along with an endogenous SpeI site 156bp upstream of the end of *brcp* were used to digest fragment (d). Subsequently, (b) and (d) were pasted together to generate the 5' arm (e) of the integration vector pLPBLP (designated in blue). For the generation of the 3' arm (f) was amplified using SL19 containing an engineered SalI site and SL20 and subcloned in an intermediate vector. A HindIII site existing on the MCS of the vector was used to excise the fragment and insert it in the pLPBLP vector. **(B) Schematic representation of the pLPBLP-*brcp*-3xHA-AID-Term plasmid.** AmpR: Ampicillin resistance gene BsR: Blastisidin resistance gene. LoxP sites are also illustrated. PshAI and PmlI restriction sites were used to excise digest the fragment that was subsequently used for transfecting *Dictyostelium* cells [sites are displayed in light blue both in pLPBLP and magnification of the inserted sequences (B) but also the endogenous locus (A)]. A magnification of the region containing the *brcp* homologous sequences is also displayed.

After a targeted integration event, the targeting construct will recombine with the endogenous sequence via HR. The resulting product will be the endogenous *brcp* gene tagged with 3xHA-AID and the blasticidin resistance gene (Figure 5.2A). After 15 days of growing under blasticidin selection, resistant clones were screened by PCR to verify targeted insertion at the *brcp* site. In detail, clones were screened either with a primer annealing to the *brcp* sequence, before the integration region (SL14) and an HA-specific primer (SL27), or with a AID-specific (SL35) and a primer annealing to the 3' sequence downstream of *brcp* (SL20) (Figure 5.2A). In the case of successful integration, PCR products of the expected size were detected, 1990bp for the 5' screen and 1820bp for the 3' screen, while Ax2 genomic DNA was used negative control for the reaction. A 600bp fragment of *brcp* was amplified (using SL10-SL06) from all templates as a positive control (Figure 5.2B). Clones were initially screened in pools of 7. Clones belonging to pools positive for the insertion were then screened individually (Figure 5.2B). The percentage of positive clones with targeted integration exceeded 90%. The above were considered sufficient evidence that clones containing *brcp-3xHA-AID* have been obtained.

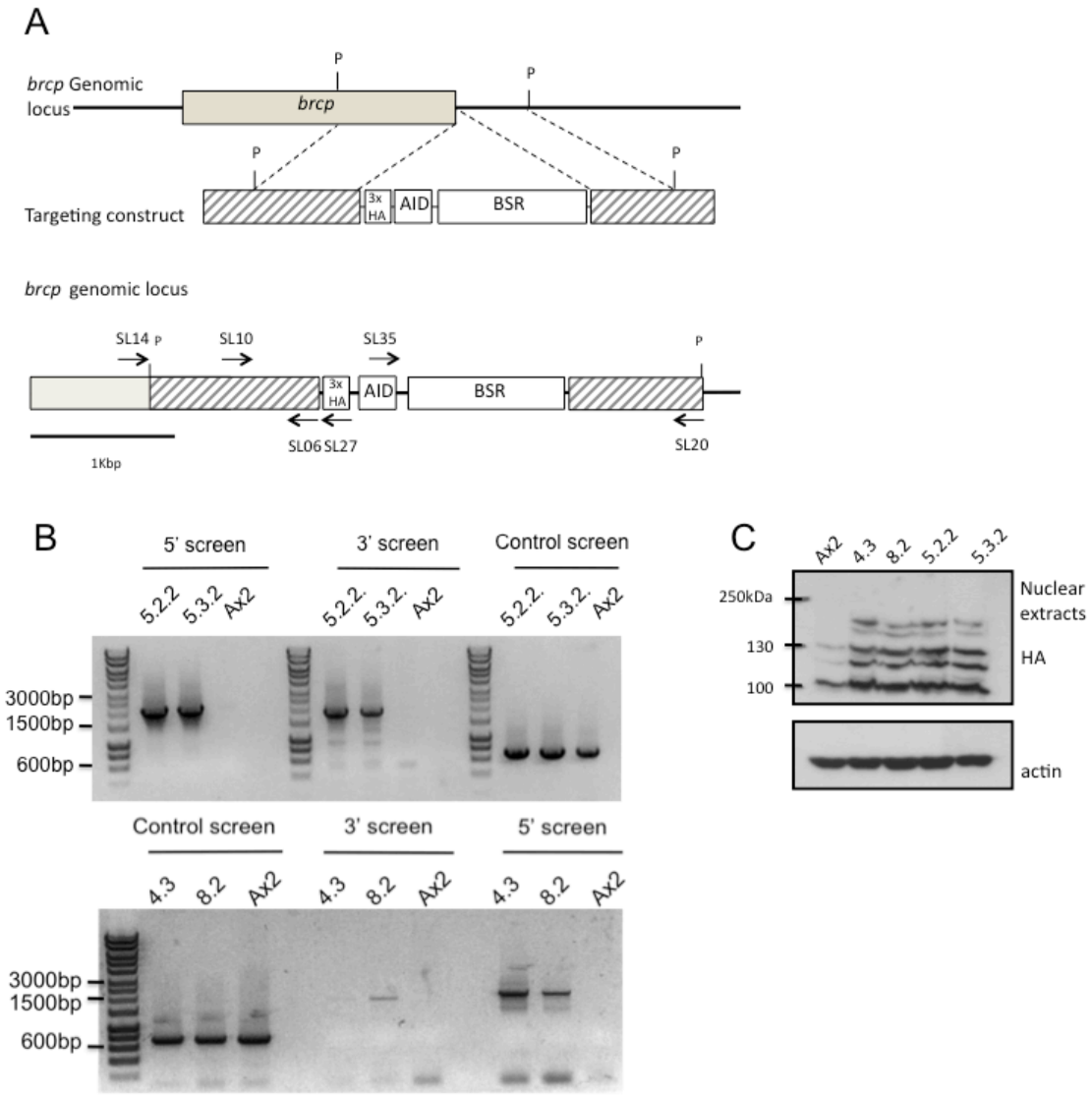

Figure 5.2: Generation and verification of *brcp*-3xHA-AID knock in strain. (A) Schematic representation of the *brcp* genomic locus in the parental and insertion strains (top and bottom respectively), as well as of the targeting construct. Hatched boxes represent the regions of homology used in the knock in construct. The insertion strategy leads to the replacement of the stop codon of the endogenous *brcp* coding sequence and the placement of three hemagglutinin tags (HA) and the AID tag followed by a short terminator sequence. (B) Integrants were screened by PCR with SL14-SL27 (5' screen), SL35-SL20 (3' screen) and SL10-SL06 (control screening). Screening of four successful clones is shown. (C) Nuclear extracts were prepared from $2x10^8$ cells. Expression of the fusion protein was verified by Western blotting using an HA monoclonal antibody, for all of the four strains generated.

Subsequently, to verify expression of Brcp-3xHA-AID, nuclear extracts derived from four integrants were analyzed by western blotting using a monoclonal HA antibody. The recombinant protein has an expected molecular weight of 213 kDa. Of the multiple bands present, the two top bands are absent in the Ax2 derived extract, which serves as a negative control. Also, the position of the top band is closer to the 250 kDa molecular marker band, something that indicated that it potentially represents the recombinant protein. Therefore, I conclude that these bands represent the fusion protein and potentially some degradation product, whereas all the other bands resulted from non-specific binding of the antibody.

5.2.2 Removal of the BSR cassette from *brcp-3xHA-AID* clones.

In order to utilize blasticidin selection for subsequent experiments that require transfection with selectable plasmids, I induced removal of the BsR cassette through transient expression of the Cre recombinase. In *brcp-3xHA-AID* clones, the BsR cassette is flanked by two LoxP sites. Transient expression of Cre induces recombination between the two sites and the BsR cassette is removed (Figure 5.3A).

Initially, the 4.3 clone (Figure 5.2C) was utilized for subsequent experiments and selected for removal of the BsR cassette. The clone was transfected with a plasmid expressing Cre recombinase and bearing a gene conferring resistance to G418. Upon transfection, cells were left to recover for one day and were selected with G418 for 1 week to express the Cre recombinase, which will induce recombination between the two LoxP sites. Selection was then removed and cells left to grow for 5 days, to lose expression of Cre. Surviving clones

were replica plated into three plates. One contained growth medium with blasticidin, the other G418 and the last no selection. Cells that were sensitive to both antibiotics but grew normally in plain medium were selected as cells contain neither the BsR cassette, nor the plasmid bearing Cre and G418 resistance. Subsequently clones were screened with primers specific to the BsR cassette and the *brcp* gene (SL10-3'AMC). In the case where BsR cassette is removed, this reaction will not yield a product. However in the parental strain, where the BsR is still present, we expect to see a product of 2000bp. Indeed, when gDNA (genomic DNA) of the parental strain (4.3) was used a band of the expected size was obtained. In the case of strains where the BsR cassette has been removed, no PCR product is observed. Amplification of a small segment of *brcp* (using SL10-SL06) from all gDNAs used served as a positive control (Figure 5.3B). The above indicate that the BsR cassette is no longer present in tested clones.

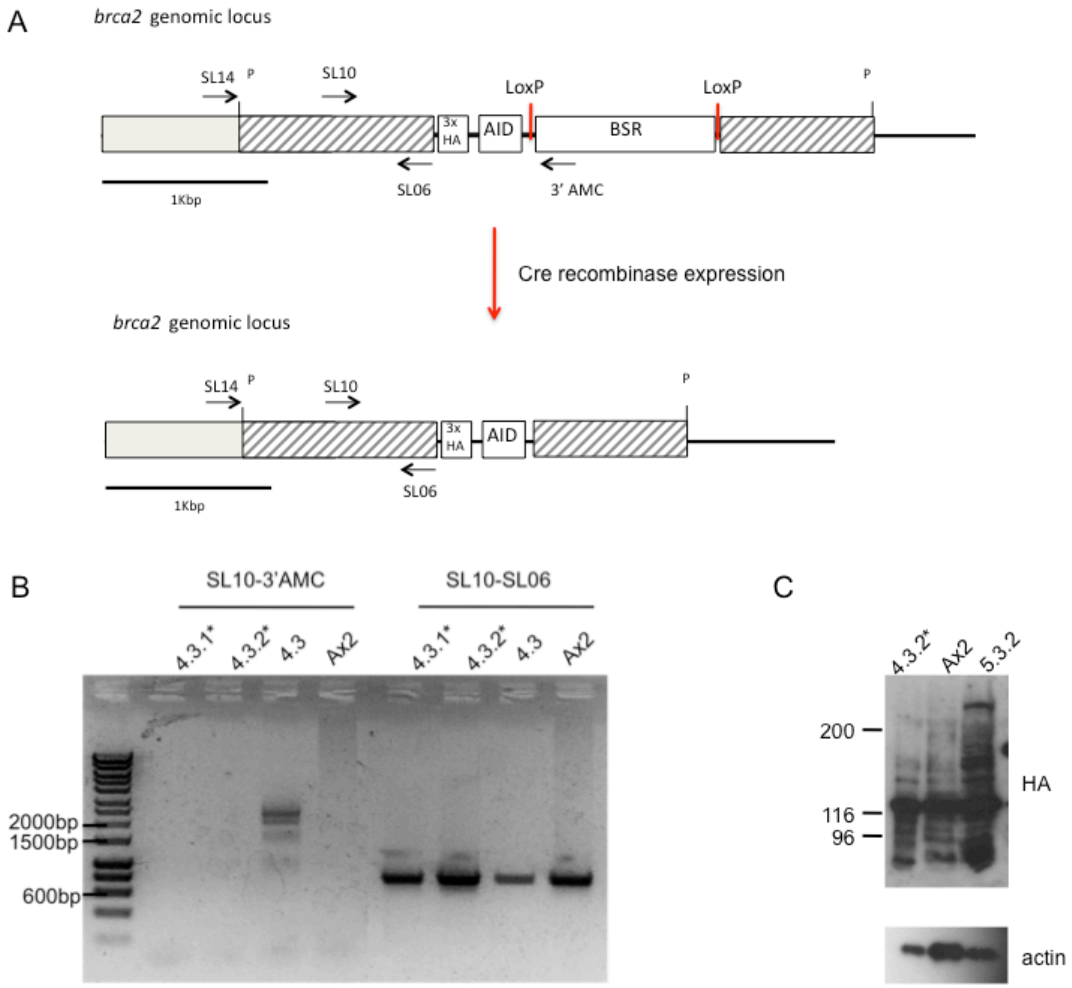

Figure 5.3: Removal of the blasticidin resistance cassette from transgenic cell lines and strain verification. (A) Removal of the BsR cassette by transient expression of Cre recombinase. (B)The screening is based on the absence of a band in the Cre loxed strains when screened with SL10-3'AMC primers(shown in A). PCR with SL10-SL06 served as a positive control. In strain where the BSR cassette is present, a band of the expected size appears as in 4.3 lane. Asterisks mark the strains where the BsR cassette has been removed. When the BSR cassette is absent the reaction doesn't give any products. (C) Nuclear extracts of the indicated cell lines were analyzed by western blotting using an HA monoclonal antibody. Actin serves a marker for equal loading and the Ax2 cell line as a negative control.

Next, I wished to test whether the Brcp-3xHA-AID is expressed in the strains where the BsR cassette has been removed. However, as illustrated in Figure 5.3C, while expression of Brcp-3xHA-AID is retained in the strain still containing BsR (5.3.2), expression is lost in the strain where the BsR cassette has been removed (4.3.2*). Ax2 extract served as a negative control for Brcp-3xHA-AID expression. Based on the above, I concluded that the recombination event might have influenced sequences flanking the LoxP sites, hence expression of the HA-tagged version of the protein was lost. This technique has so far been used in our laboratory for removing the BsR cassette from disruption strains, where the integrity of the sequences flanking the BsR cassette is not as important, since the gene of interest is not functional anymore [123]. To overcome the problem, the 5.3.2 strain, still containing the BsR cassette, was used for all subsequent experiments.

5.2.3 Study of Brcp function in *Dictyostelium*.

Having established a strain carrying an AID-tagged version of Brcp, I wished to utilize it to study the function of the protein *in vivo*. In order to achieve this, two different approaches were used. The HA tag allows for detection of the Brcp-3xHA-AID by well established biochemical techniques and allows to assess whether upon damage induction, Brcp gets enriched in the chromatin fraction. Subsequently, I wished to utilize the AID degron system (see chapter 4) to degrade Brcp-3xHA-AID and assess whether its depletion has an impact on *Dictyostelium* cell viability and the ability of *Dictyostelium* cells to perform DSB repair.

5.2.3.1 Brcp gets recruited to chromatin upon DSB induction

To determine whether Brcp is recruited to chromatin upon DSB induction, Brpc-3xHA-AID expressing cells were treated with phleomycin for various times. Chromatin extracts were prepared and subsequently analyzed by western blotting (Figure 5.4). A clear and gradual enrichment of Brcp-3xHA-AID, a protein of 213 kDa, can be observed upon induction of DSBs (Figure 5.2C). A second band is also visible and is believed to be a degradation product of the full-length protein. The protein is recruited to chromatin 30 minutes after induction of DSBs and its amount gradually increases until 120 minutes, where it peaks. From this point onwards, the signal decreases, implying a potential dissociation from chromatin. These data imply, that Brcp has a role in the repair of DSBs. When compared to Ku80, the kinetics of recruitment of Brcp are not dramatically different. However, the Brcp signal seems to peak at a later time point compared to Ku80. This differential recruitment could indicate different roles for the two proteins in the repair process. We speculate Brcp might be implicated in HR in *Dictyostelium* due to its domain structure. However, further verification of this observation is required. To that end, I proceeded to deplete cells of Brcp to study potential phenotypes arising.

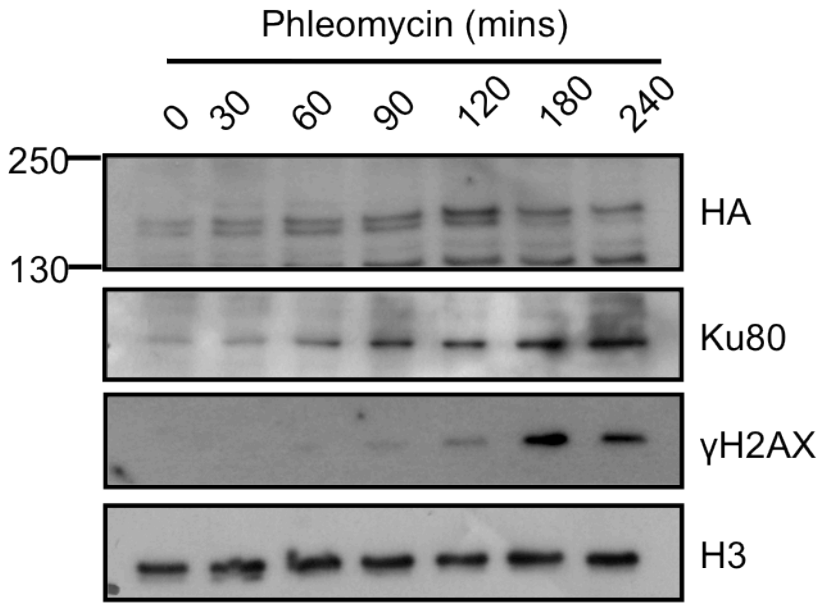

Figure 5.4: Recruitment of Brcp to chromatin upon DSB induction. $2x10^8$ cells were treated with phleomycin for increasing times up to 240 minutes and then used for preparation of chromatin extracts. Extracts were analyzed by western blotting, using the indicated antibodies. The HA antibody was used to detect Brcp-3xHA-AID, H3 served as a loading control and γH2AX as a marker of DSBs.

5.2.3.2 Rapid degradation of Brcp via the auxin degron system.

It has already been established that an AID-tagged protein expressed from an extrachromosomal vector in osTIR expressing cells could be depleted upon auxin treatment (see Chapter 4). I wanted to test whether I could similarly deplete the endogenous Brcp-3xHA-AID. Accordingly, Brcp-3xHA-AID cells were transfected either with the pDXA-3C-osTIR plasmid or with empty pDXA-3C vector. Both strains were treated with auxin for increasing periods of time, up to 6 hours. Upon treatment, nuclear extracts were prepared and loss of Brcp-3HA-AID assessed by Western blotting (Figure 5.5).

A distinct band can be detected with a monoclonal HA antibody at the predicted size (213 kDa) in the 5.3.2 cells expressing osTIR, whereas no signal is detected from control Ax2 cells. Moreover, when cells are treated with auxin for increasing times, the signal is diminished, indicating the degradation of the Brcp-3xHA-AID protein over time. This result is in agreement with the findings in other organisms, where endogenous proteins have been fused with the AID tag and subsequently degraded in cells expressing osTIR inducted with auxin [147].

The above data indicate that endogenous AID-tagged proteins can be specifically and rapidly degraded upon auxin treatment in an osTIR-dependent manner, presenting a unique opportunity for use of this system to study proteins essential for cell viability in *Dictyostelium* cells.

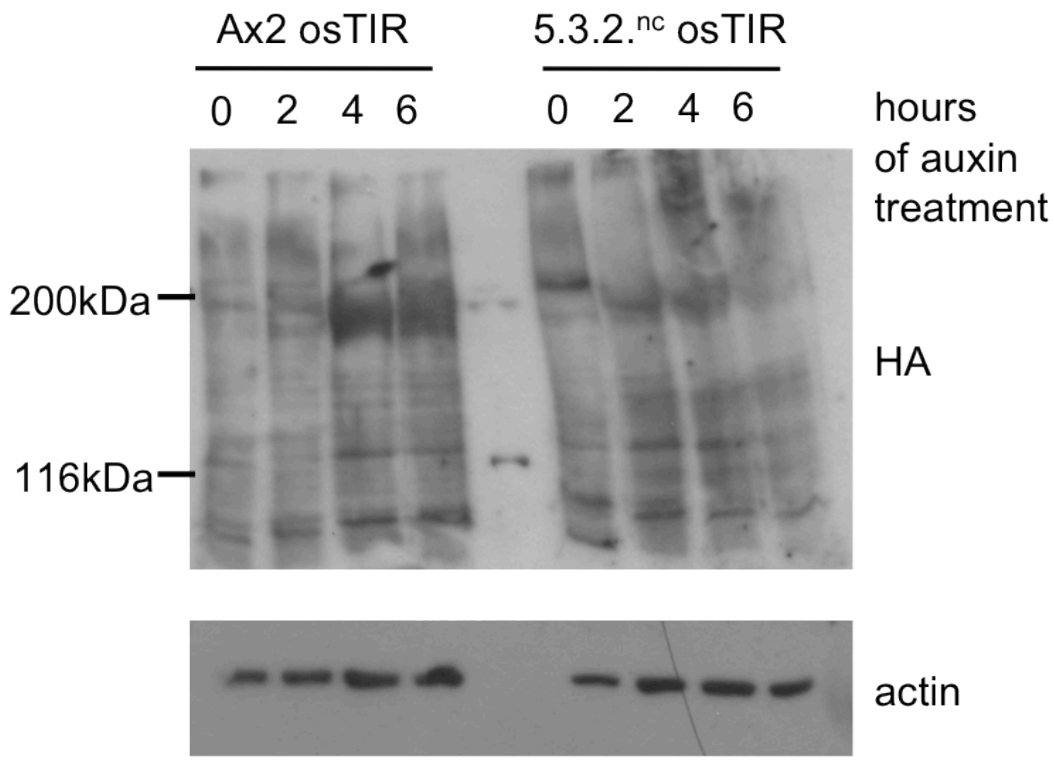

Figure 5.5: Auxin inducible degradation of Brcp. 2 x 10^8 cells were treated with auxin for the indicated times. Subsequently, nuclear extracts were prepared and analyzed by western blotting using the indicated antibodies.

5.2.3.3 Brcp depletion is not toxic for *Dictyostelium*.

Several lines of evidence suggest that BRCA2 inactivation in various organisms is lethal [70, 88]. In agreement with the above, when disruption of *brcp* in *Dictyostelium* was attempted by us and another group [113], it was unsuccessful. All the above suggest that Brcp might be essential for cell viability in *Dictyostelium*. In an attempt to officially test this hypothesis, I depleted Brcp from *Dictyostelium* cells and assessed the impact of this depletion on cell viability. To achieve this, I utilized a cell viability assay whereby Ax2 and Brcp-3xHA-AID cells expressing either osTIR or empty pDXA3C vector, were treated or not with auxin over the course of five days. At discrete time points cell viability was measured using the trypan blue assay, which is based on the exclusion of the trypan blue dye from live cells. Dead cells, stained blue, were counted and scored relative to live cells. When percentage of mortality was plotted on a graph, I observed increasing cell death over time with no significant difference between the examined cell lines. The above data indicate that Brcp-3xHA-AID cells expressing osTIR, treated with auxin do not display reduced viability due to the depletion of Brcp under these assay conditions.

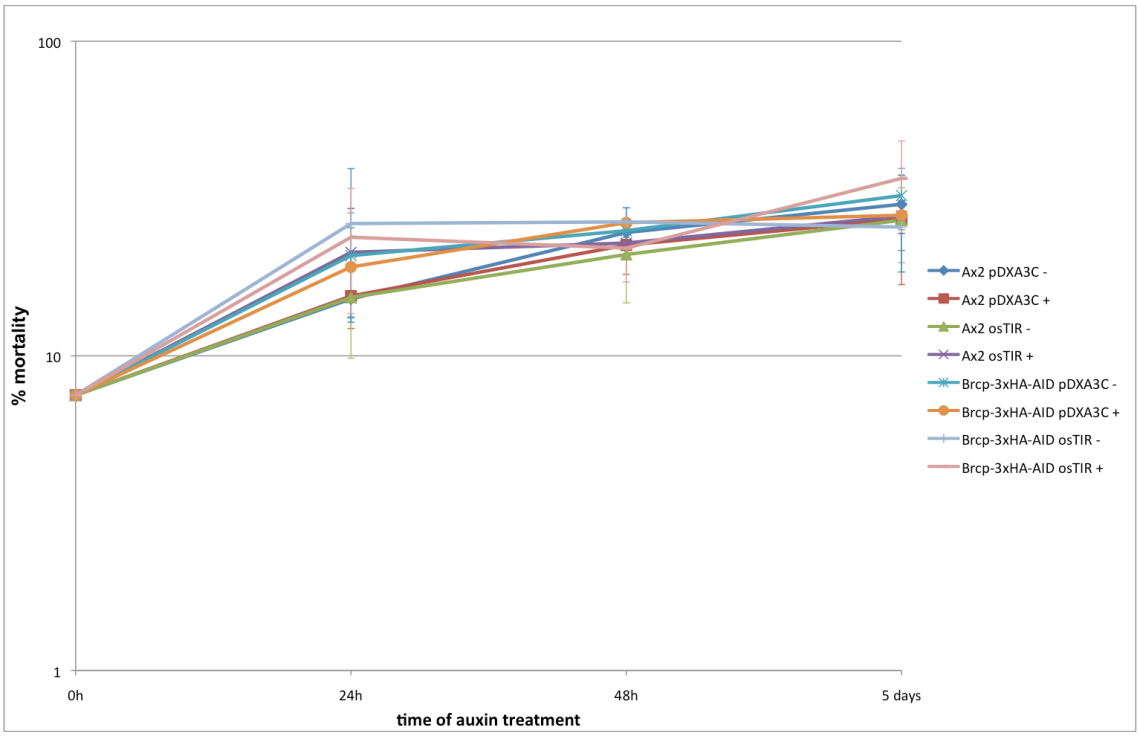

Figure 5.6: Brcp is not essential for cell viability. Cells from the indicated cell lines treated with auxin (+) or not (-) were seeded and treated with 0.5mM auxin or water, respectively, at a density of 1 x 10^6 cells/ml and counted. Subsequently, cells were grown for 5 days and split down to the same density every day and supplemented with auxin. Cell viability was measured at the indicated time points.

5.2.3.4 Brcp depleted cells might be sensitive to induction of DSBs

Since BRCA2 has been shown to have a central role in DSB repair in various organisms [74, 86-90], I wanted to test whether the same is true for *Dictyostelium*. To this end, I tested the ability of Brcp-3xHA-AID cells to perform DSB repair by inducing this type of damage and assessing cell viability relative to untreated cells. In the experiment conducted, Brcp-3xHA-AID osTIR expressing cells (Brcp-3xHA-AID osTIR) as well as control Ax2 osTIR cells were pre-treated or not with auxin for 3 hours. Subsequently, phleomycin was added for another hour to induce DSBs. 200 cells of each sample were then platted on a *Ka* lawn and viability was assessed by counting plaques formed after 3 days of growth. This gave me the opportunity to examine both whether auxin had a non specific effect on cell viability in cells not expressing AID-tagged proteins (Ax2 osTIR), but also to monitor cell viability of the Brcp depleted cells upon damage induction (Brcp-3xHA-AID osTIR +, Figure 5.6).

Our results show that there is no significant difference in sensitivity to DSB induction between the tested cell lines. Increased sensitivity of Brcp-depleted cells could not be verified by this set of experiments and due to time constraints could not be repeated. In the future, it would be interesting to further test whether the above cell line shows increased sensitivity to DSB induction.

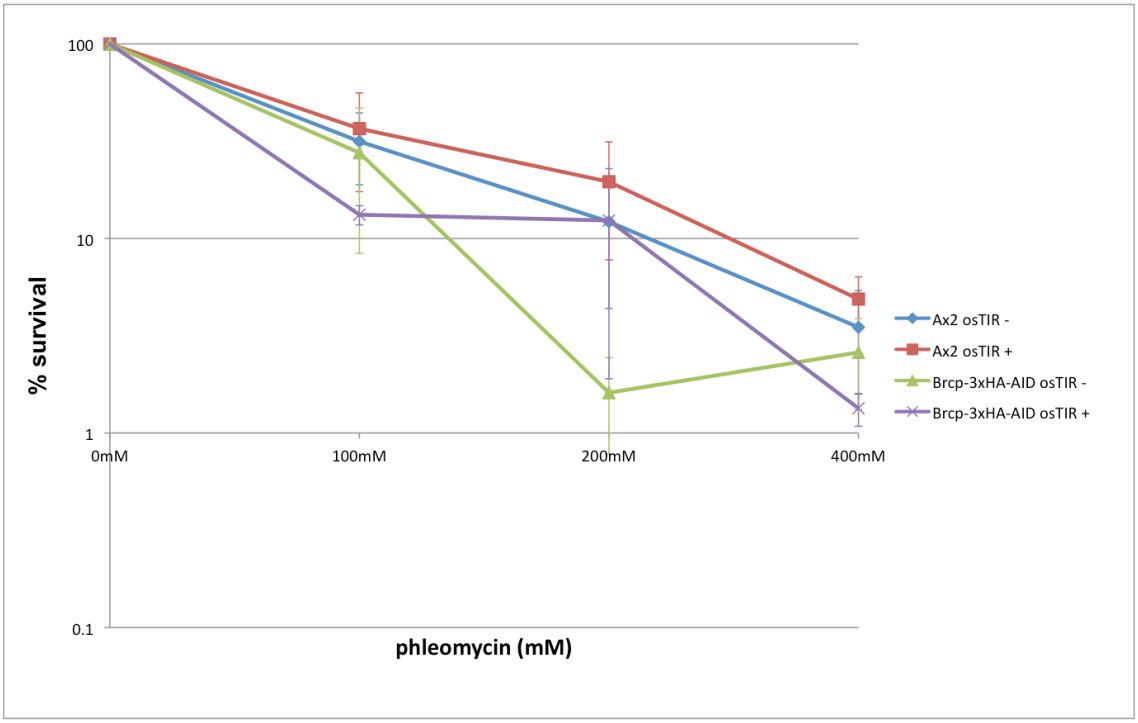

Figure 5.7: Brcp-3xHA-AID cells treated with auxin are more sensitive to induction of DSBs. Both Ax2 and Brcp-3xHA-AID cells expressing osTIR, pre-treated (+) or not (-) with 0.5mM auxin for 4 hours, were treated with 400µg/ml of phleomycin for one more hour at a density of $1x10^6$ cells/ml. Cells were then diluted in KK2 to a density of 10^4 and of those, 200 were plated, along with KK2-*Ka* on SM agar plates. Cells were left to grow for three days and plaques forming were scored for the three following days. Values are represented as percentage survival compared to the 0 concentration of phleomycin for each cell line. The mean percentage of three experiments has been scored in this graph.

5.3 Discussion

BRCA2 is a protein essential for genome stability and its mutation in human cells causes gross chromosomal rearrangements [73, 74]. As shown from studies in humans and other organisms, BRCA2 is essential for HR-mediated repair of DSBs [74, 86-90]. Identification of a BRCA2 ortholog in *Dictyostelium* and an in depth bioinformatics search, showed that the protein identified displayed a domain structure similar to other BRCA2-related proteins [87-90]. This finding encouraged me to study the function of Brcp in *Dictyostelium* cells. The embryonic lethality caused by deactivation of BRCA2 in other organisms [70, 88] combined with failed attempts to generate a *brcp* disruption strain in *Dictyostelium* (this study and [113]) favored the hypothesis that Brcp might be essential for cell viability. As an alternative route to characterize Brcp, I established a conditional protein depletion system, the AID degron system (see chapter 4). To utilize this system to deplete Brcp I first generated a strain containing an HA and AID-tagged version of the endogenous Brcp.

This enabled me to use two different approaches to define the cellular function of Brcp. Using the HA tag to detect endogenous Brcp, I observed that Brcp is gradually recruited to chromatin upon DSB induction, a result that implicates it in DSB repair in *Dictyostelium* (Figure 5.4). The kinetics of recruitment, when compared to an NHEJ protein, Ku80, are slightly different, with Brcp levels peaking at later time points after DSB induction. Due to its domain structure showing similarity to that of BRCA2-related genes, it is possible that Brcp functions in HR in *Dictyostelium*. However, further experiments are required to

clarify the role of Brcp in DNA repair in *Dictyostelium.* The main avenue through which its role in live cells could be explored, is its depletion.

I have managed to conditionally deplete Brcp from cells using the AID degron system (described in chapter 4). Specifically, a strain expressing osTIR and carrying a version of the endogenous Brcp a C-terminal fusion of 3xHA tags and the AID tag was treated with auxin, to ultimately deplete the Brcp protein (Figure 5.5). Depletion is achieved within two hours of treatment and is sustained for up to six hours. These findings show that the auxin degron system can be successfully used to deplete endogenous proteins carrying the AID tag, when osTIR is expressed and cells are treated with auxin. This result is consistent with previous results obtained form the use of the system in cells derived from other organisms (such as yeast, chicken, mouse, human) [147]. In these experiments, endogenous proteins essential for vital processes (e.g. MCM4 a protein important for DNA replication, CENP-H a protein essential for the formation of the kinetochore) were depleted causing cell cycle arrest and eventual cell death [147]. Although such extreme phenotypes were not observed in the case of Brcp depletion, I confirmed by western blot analysis that the protein is degraded after two hours of treatment with auxin. This observation has been made for the proteins mentioned above in different cell systems [147].

The above mentioned strain was utilized to study potential phenotypes that may arise from depletion of Brcp. Upon treatment of cells with auxin, their viability did not show a significant reduction compared to control cell lines. The above result contradicts our hypothesis that disruption of Brcp might be lethal. However, the context of the two observations is different and the result should

be interpreted carefully. Although no cell death can be observed in the context of the trypan blue viability assay, we cannot exclude the possibility of gross chromosomal rearrangements being induced within these cells. This observation has been previously made in human cells, where BRCA2 depletion or mutation causes gross chromosomal rearrangements and DNA damage checkpoint activation [73]. It will be interesting to examine whether depletion of Brcp causes checkpoint activation in *Dictyostelium* cells and whether aberrant chromosome structures arise upon depletion of Brcp. This chromosomal instability would not lead cells to immediate cell death, something that might explain the phenotype observed. However, in the case of a disruption strain, cells must undergo numerous cell divisions before they can be used for experiments. After several generations have passed, genomic instability caused by the absence of Brcp might become toxic and lead to cell death.

It would be interesting to study whether induction DSBs in Brcp-depleted cells are less capable of repairing this type of damage. Data from this study show that this is not the case (Figure 5.7), but repetition of the experiment could wield different results. Due to time constraints, an other data set of this experiment could not be obtained. Further experiments could indicate whether Brcp is implicated in DSB repair and whether its depletion causes defects in repair of exogenously induced DSBs. This increased sensitivity to DSB inducing agents would be consistent with findings in mice, where Brca2 deficient animals exhibit extreme sensitivity to DSB inducing agents [70, 74]. The same is true for human cells defective in BRCA2 [73].

From the above we can conclude that although transient depletion of Brcp does reduce cell viability of vegetatively growing *Dictyostelium* cells, it might play a role in radiation resistance and repair of DSBs in the organism. Further experiments must be conducted to elucidate the function of the protein in *Dictyostelium* cells. An interesting experiment to be conducted is measurement of the efficiency of targeted integration at the *cdk8* locus. This assay is used as a readout for efficiency of HR in *Dictyostelium* cells, as targeted integration is reduced in HR deficient mutants such as *exo1*$^{-}$ cells [110]. Also, to investigate whether Brcp has a role in HR specifically as a mediator of Rad51, as shown in other organisms [79, 88, 91], it would be interesting to conduct co-immunoprecipitation experiments to identify *in vivo* partners of the protein.

A synthetically lethal relationship exists between PARP1 and BRCA2 inactivation in human cells. Inhibition of PARP1 by use of inhibitors such as NU1025 in a mutant BRCA2 background, results in specific killing of BRCA2 deficient cells [98]. In *Dictyostelium*, we have found PARPs to be conserved and implicated in DSB repair [112]. If a synthetically lethal relationship can be observed between these proteins and Brcp, *Dictyostelium* could be used as a model for the study of the molecular basis of this synthetically lethal relationship. A technique for random mutagenesis in *Dictyostelium* termed restriction enzyme-mediated integration (REMI) [158] could be used to generate mutants in a Brcp-AID background. Upon induction of degradation of Brcp and treatment with PARP inhibitors, surviving mutant colonies could be further tested for the basis of their resistance. It is highly probable that some of these genes will have human orthologs, since, as revealed from the present study as well as data published when the *Dictyostelium* genome was sequenced, the

organism shows a great degree of conservation in pathways related to human disease [103]. The studies proposed above could reveal new targets for cancer therapy that could confer resistance to treatment of patients with PARP inhibitors.

Chapter 6: Discussion

In this study, we addressed the conservation of the HR pathway in *Dictyostelium*. Through a bioinformatics search, I was able to identify putative orthologs of most known human counterparts of proteins involved in HR repair. Although the putative orthologs have not been experimentally verified, previous findings regarding pathway conservation from humans to *Dictyostelium* are encouraging. Many pathways involved in human disease have been shown to be conserved in *Dictyostelium* including G-protein coupled receptor mediated signaling and phosphotyrosine signaling [103]. Focusing on DNA repair pathways, NHEJ, which is believed to be the main pathway for repair of DSBs in human cells, is conserved in *Dictyostelium* and is important for resistance to DSBs at certain developmental stages [106]. Most importantly, key components of this pathway, such as the PIKK kinase DNA-PKcs and the nuclease Artemis, which were previously thought to be metazoan-specific are conserved in *Dictyostelium* [109, 110]. Another interesting finding is that *Dictyostelium* have a minimal FA complex, which is involved in interstrand crosslink repair [113]. Interestingly, in other lower eukaryotes such as budding and fission yeast the FA pathway is not conserved. All the above indicate that *Dictyostelium* is a good model to study DNA repair pathways as it shows more similarities to humans compared to other lower eukaryotes currently used as models for DNA repair. To further support this hypothesis, I investigated conservation of the HR pathway in the organism.

Previously conducted experiments in *Dictyostelium* have shown that mutation *exo1*, a nuclease implicated in DNA end resection, causes greatly reduced HR efficiency in vegetatively growing *Dictyostelium* [110]. This finding is consistent with those in yeast, where Exo1 is required for proficient HR [159]. We wished to test other putative HR protein orthologs for conserved functions.

Initially, I decided to study the role of a putative ortholog of RAD51 that was identified in *Dictyostelium*. Rad51 is a recombinase that functions to mediate homology search and strand exchange in human cells and is essential for the function of the pathway. Vertebrate cells lacking Rad51 display genome instability and subsequently die [54]. Equally, targeted inactivation of Rad51 in mice results in early embryonic lethality [160]. Although the molecular function of Rad51 in *Dictyostelium* has not been studied, a study concludes that Rad51 depletion results in cell death [151]. To study the role of the *Dictyostelium rad51-2*, a protein that shows 74% overall identity to the human RAD51, I developed a subcellular fractionation technique to monitor the changes in localization of DNA repair proteins when DSBs are induced. In proof of principle experiments I conducted, I was able to demonstrate that Ku80, a NHEJ protein, gets enriched in the chromatin fraction upon DSB induction while its expression levels stay constant. Moreover, I have proven that the recruitment is time and dose-dependent. In conclusion, the method developed allows form monitoring of changes in DNA repair protein recruitment to chromatin upon DSB induction, which is proportionate to the amount of damage caused. At the same time it offers good time resolution displaying changes in protein levels with increasing time of treatment with the damaging agent.

Comparing the kinetics of recruitment of Ku80 to Rad51 I found that the latter gets recruited to chromatin and its amount reaches saturation at later times compared to the former in vegetative *Dictyostelium* cells. Vegetative *Dictyostelium* cells are predominantly in G2 phase of the cell cycle [114] and utilize HR for resistance to DSBs [110]. The observation that *rad51-2* gets recruited to chromatin upon DSB induction suggests it operates in repairing DSBs, potentially through facilitating HR. These results are consistent with findings derived from other model organisms, which show that Rad51 operates in HR, with its function being indispensible for cell survival [54, 160]. Its requirement for *Dictyostelium* viability [151] combined with its gradual enrichment on chromatin upon DSB induction, could indicate a role for *rad51* in HR and maintenance of genome stability. In the future conditional depletion experiments with *rad51* could help define its role in HR in *Dictyostelium* as well as the role of HR in repair at different phases of *Dictyostelium* growth. These studies will help us define similarities and differences in HR utilization between *Dictyostelium* and other organisms, giving new insight to the evolution of this pathway of DNA repair.

With the above experiments implying that Rad51 is implicated in DSB repair in *Dictyostelium,* I wanted to examine another interesting finding of the bioinfomatics search conducted; namely, the identification a putative BRCA2 ortholog in the *Dictyostelium* genome. BRCA2 is a protein indispensible for HR in humans, whose absence or inactivation causes genome instability and predisposition to cancer [68, 73, 161]. The putative ortholog identified, named Brcp*,* possesses conserved domains found on the human protein and all identified BRCA2-related proteins from other organisms, such as mice, chicken,

C.elegans and the fungus *Ustilago maydis* [77, 78, 87-90]. More specifically, Brcp contains three BRC repeats, domains unique to the BRCA2 protein [77]. The BRC repeats of the human protein mediate its interaction with Rad51 *in vitro* and *in vivo* [79, 81, 91, 95, 162, 163]. The number of repeats found in BRCA2-related proteins varies from organism to organism [78, 86, 87, 89, 90], with the human protein containing the most (8 repeats) [77] while *C.elegans* only containing one repeat [88]. This one repeat is required and sufficient to mediate CeBRC-2's role in HR [88]. Although the BRC repeats found in *Dictyostelium* are shorter than those found in other organisms, the amino acids identified to be important for interaction with RAD51 through structural studies, are conserved [91]. Other conserved domains on Brcp are a helical domain and two OB1 folds, which have been found to recognize and bind ssDNA in the human protein [85]. All the above suggest that Brcp is highly likely to be an ortholog of BRCA2.

The above findings encouraged me to study the function of Brcp and its possible role in repair of DSBs by HR in *Dictyostelium* cells. To this end, I first attempted to generate a strain disrupted in *brcp*. Although more than 300 colonies were screened, no successful disruption clones were identified. Disruption of this particular gene has been attempted by a different group, but was similarly unsuccessful [113]. These two lines of evidence suggest that disruption of the aforementioned gene is challenging since its function might be essential for cell viability. To circumvent this problem, I decided to establish a system that would conditionally deplete cells of the *brcp* gene product. A few systems are available in *Dictyostelium*. A tetracycline-inducible system, as well as an inducible RNAi system can be used for conditional expression or

depletion of proteins [144, 145]. In both cases, induction of the system takes several hours and their efficiency is limited by several factors described below. In the case of RNAi, it is possible that the hairpin generated will not reduce expression efficiently. Equally, induction of expression or repression by tetracycline takes several hours and reversal of the drug's effect (repression or induction of expression) also requires time. In order to study gene function in cases where gene products are essential for cell viability, a system that would deplete the protein of interest rapidly and reversibly is required. To that end, we chose to establish a system which had already been tested in other cell culture system (human, mouse, avian and yeast cells). This system is based on a plant specific response to the growth hormone auxin, whereby members of the IAA transcriptional repressors' family are degraded by the SCF E3 ubiquitin ligase complex, when recognized by the TIR1 mediator in the presence of auxin. When auxin is absent, the proteins are expressed normally. Transplantation of all the essential components of the system in a heterologous system, results in rapid and reversible degradation of a target proteins [147].

I initially tested the functionality of the AID degron system by expressing GFP fused to the AID tag and osTIR, extrachomosomally. Upon induction of the system with auxin a clear osTIR-dependent reduction of GFP-AID levels can be observed (Figures 4.8 and 4.9), although the protein is not completely depleted, perhaps due to its overexpression. This result shows that the system can be used in *Dictyostelium* to conditionally deplete an appropriately tagged protein.

The above data encouraged me to apply the system to the endogenous Brcp protein. Accordingly, I created a strain where the endogenous *brcp* sequence

has been fused to 3xHA tags and the AID tag. The strain generated was initially utilized to study the cellular function of Brcp. Utilizing the HA tags, I examined whether the protein gets recruited to chromatin upon the induction of DSBs. Indeed, I can observe recruitment of Brcp to chromatin in a time-dependent manner upon damage induction (Figure 5.4), something that implies a role for Brcp in DSB repair in vegetative *Dictyostelium.* This observation is consistent with recruitment of Brca2 to chromatin post-DSB induction initially observed in mice via indirect immunofluorescence [71]. However, further experiments are required to verify a role for Brcp in DSB repair and more specifically in HR.

The main way to explore the implication of Brcp in repair pathways is through its depletion. To this end, I utilized the AID degron system to conditionally deplete Brcp from cells. Specifically, a Brcp-3xHA-AID strain expressing osTIR was treated with auxin. Upon two hours treatment, the protein levels are diminished (Figure 5.5). This result is particularly important for future research in the *Dictyostelium* community, as I have shown that a fusion of an endogenous protein with the AID tag, along with expression of osTIR and induction of the system with auxin can result in conditional depletion of the tagged protein to non-detectable levels. This technique can be utilized to study the cellular function of proteins that are essential for cell viability, a case in which gene disruption is not possible. At the same time, this system presents an advantage when compared to the other conditional systems previously used in *Dictyostelium* (and described above) in that the depletion of the protein of interest is rapid and happens within two hours of treatment with auxin.

Taking advantage of the system, I sought to study any phenotypes that arise from the depletion of Brcp. To examine whether cell death was induced in the absence of Brcp, I conducted a cell viability assay over the course of five days, while continuously treating the cells with auxin. When compared to control cell lines, the Brcp-AID, osTIR-expressing, auxin-treated cells, do not show reduced viability (Figure 5.6). This observation shows that depletion of the protein for a short time does not lead to cell death of exponentially growing cells. This result, does not exclude the possibility *brcp* disruption might result in eventual cell death after numerous cell divisions, due to genome instability causing aberrant chromosome structures and extensive damage. In other organisms, depletion of BRCA2-related proteins causes genome instability associated with defects HR [73, 87] in many cases leading to embryonic lethality [70, 88]. Future experiments in *Dictyostelium* utilizing methods such as chromosome spreads and time lapse microscopy using fluorescent cell cycle markers could help determine whether Brcp depletion causes chromosomal rearrangements or cell cycle arrest, respectively [114, 164].

Finally, to determine whether Brcp has a role in DSB repair, Brcp-depleted cells were assessed for their ability to tolerate DSBs. When compared to control strains, Brcp-depleted cells do not display a significantly greater sensitivity to DSB induction (Figure 5.7). Due to time constraints, the experiment could not be repeated and the result validated. In the case this observation is true, the phenotype observed is not as dramatic as the one observed in human and mouse cells when BRCA2 was first implicated in DNA repair [74]. However, we cannot argue that Brcp is indeed an ortholog of BRCA2 unless further experiments are conducted. To test whether Brcp is implicated in repair via HR,

targeted gene integration experiments need to be conducted in the Brcp-depleted strain, which will show whether these cells are proficient in HR repair [110]. To elucidate the molecular function of Brcp, immunoprecipitation experiments could offer insight to whether Brcp is indeed a partner of Rad51 in *Dictyostelium*. Another interesting experiment to be conducted in the future would be to assess whether Brcp depleted cells are sensitive to PARP inhibitors (PARPi). In human cells, inhibition of PARPs or more specifically PARP1, causes rapid and specific cell death of cells mutated in BRCA2, presenting a unique opportunity for the use of PARP inhibitors as anti-cancer drugs [98]. A synthetically lethal relationship between *Dictyostelium* PARPs and Brcp could help us study its molecular basis in this very simple unicellular organism. Conservation of PARPs is another unique feature of *Dictyostelium,* as these signaling molecules are not conserved in other lower eukaryotes. Although a BRCA2 ortholog has been characterized in *U.maydis,* the absence of PARPs would not allow the study of synthetic lethality.

In summary, in this study we have shown that most human HR proteins have putative orthologs in *Dictyostelium discoideum.* I have identified a putative ortholog of the RAD51 recombinase and shown that it gets recruited to chromatin upon DSB induction, implicating it in DSB repair in vegetative *Dictyostelium* cells. I have also identified a putative ortholog of BRCA2. The same domains are conserved in BRCA2-related proteins identified in other model organisms. To study the function of the protein, I have developed a technique to specifically and inducibly deplete endogenous proteins in *Dictyostelium*, which could be used by the *Dictyostelium* research community for study of gene function in cases where disruption is lethal. By applying this

technique I was able to deplete cells of the gene product of Brcp, which does not reduce cell viability of vegetative *Dictyostelium* but might be important for DSB repair. *Dictyostelium* is a highly tractable organism, which shows conservation of many pathways that were thought to only exist in higher eukaryotes. Conservation of numerous DNA repair pathways can be exploited to dissect the molecular basis of interactions that are hard to study in more complex organisms.

Chapter 7: Appendices

Appendix A: Primers

Primers for *brcp*

SL01
GCGGGATTCGGACAAAAATTAATTTCAGAAGAAGATTTAATGAAAAGTAATT
C

SL02
TCTACTTGTAATTTCTAGAACATC

SL03
GAATGATGTTCTAGAAATTAC

SL04
GGATTAACCATATGTAATGTTAATAAG

SL05
ATTAACATTACATATGGTTAATCC

SL06
GTGGGATCCTTACCCAGAGTTTTCCAAAAAACTGGATCG

SL05+ BamHI
AAGGATCCATTAACATTACATATGGTTAATCC

SL06 + BamHI
AAGGATCCTTACCCAGAGTTTTCCAAAAAACTGGATCG

SL07
AATTGGTACCCAAGTGATGATGATAATTCGAATG

SL08
AATTGTCGACTATCCTCGTCAGCAATGGATG

SL09
AATTGGATCCCTGGATCGAAATCGAAGTTAAAATC

SL10
AATTCTGCAGCTGATAGATCTGGTTTCATTGGTAG

SL11
AATTGGTACCATGAAAAGTAATTCAAATAAAAGAG

Sl12
AATTGTCGACCTTTAGCTTCTCTTCAGAGATTACA

SL14 (5’ screen KI)
GAATGGTATATCACCATTGGAAGAT

SL15
TACCAACTAACTCTACGTGTACTA

SL16
GCTAACCTATCATATTTCATATTTCA

SL17
AAATGGCGATTCTGATGGTGGCTC

SL18 (SalI)
AAGTCGACCCCAGAGTTTTCCAAAAAAACTG

Cloning of KI 3’ arm

SL19 (KpnI)
TTAGGTACCGAGTTTTTGACGAATTAAGAAATTG

SL20 (SalI)
ACGTGTCGACGGTTGGAATTGGGATGGTAAAC

Screening

Primers for BsR cassette

Check 1
GGATGGATCAATTTAACATTTCTC

Check2
CGGGTATATTTGAGTGGAATGAG

AMC BSR 5’ Arm
GAGAAATGTTAAATTGATCCAT

AMC BSR 3’ Arm
GGCAAGTTAGTCAAAACTAC

SL27 5’arm screen
GCATAGTCAGGAACATCGTATG

SL28 – 3’ screen
GTTAGTCAAAACTACGATTGAAG

SL35
Primer to screen from AID tag towards the BSR cassette

GATGGAGAAGTGCAAGAGCAGAGC

Rad51 cloning

SL34
ATGGATGGTACCGATTATAAAGATGATGATGATAAAATGGCATCAAGACAA
AGACAAG

SL33 (BamHI)
GGATCCTTATTGTTCTTTATAATCGGAGATacc

Terminator oligos

Oligo1_TermBRCA2
gtcaacccgggAATTTTAAATTGTTTTAAAAATAAATAGAATCATACAAATATTTT
ACTAGTAGCCTGCAGaacag

Oligo2_TermBRCA2

ctgttCTGCAGGCTACTAGTAAAATATTTGTATGATTCTATTTATTTTTAAAACA
ATTTAAAATTcccgggttgac

AID-GFP-NLS cloning

Forward Primer
SL25(KpnI)
ATGGATGGTACCATGGTGAGCAAGGGCGAGGAG

Reverse primer
SL26 (XhoI)
AGATCGCTCGAGTTAAACCTTT(?)ACGTTTCTTTTTAGGG

Appendix B: *Dictyostelium* orthologs gene accession numbers

h2ax:
DDB_G0279667

atr:
DDB_G0291380

rad50:
DDB_G0292786

mre11:
DDB_G0293546

Rfa1:
DDB_G0289513

exo1:
DDB_G0291570

CtIP ortholog:
DDB_G0291466

53BP1 ortholog:
DDB_G0286539

DNA2 ortholog:
DDB_G0274777

wrn:
DDB_G0268512

blm:
DDB_G0292130

top3:
DDB_G0275257

mus81:
DDB_G0276519

rad51-1:
DDB_G0273139

rad51-2:
DDB_G0273611

rad52:
DDB_G0269406

rad54:
DDB_G0282997

RAD51C ortholog:
DDB_G0284507

Rad51D ortholog:
DDB_G0285287

XRCC2 ortholog:
DDB_G0290297

XRCC3 ortholog:
DDB_G0283637

BRCA2 ortholog:
DDB_G0293414

Appendix C: Supplementary data

A

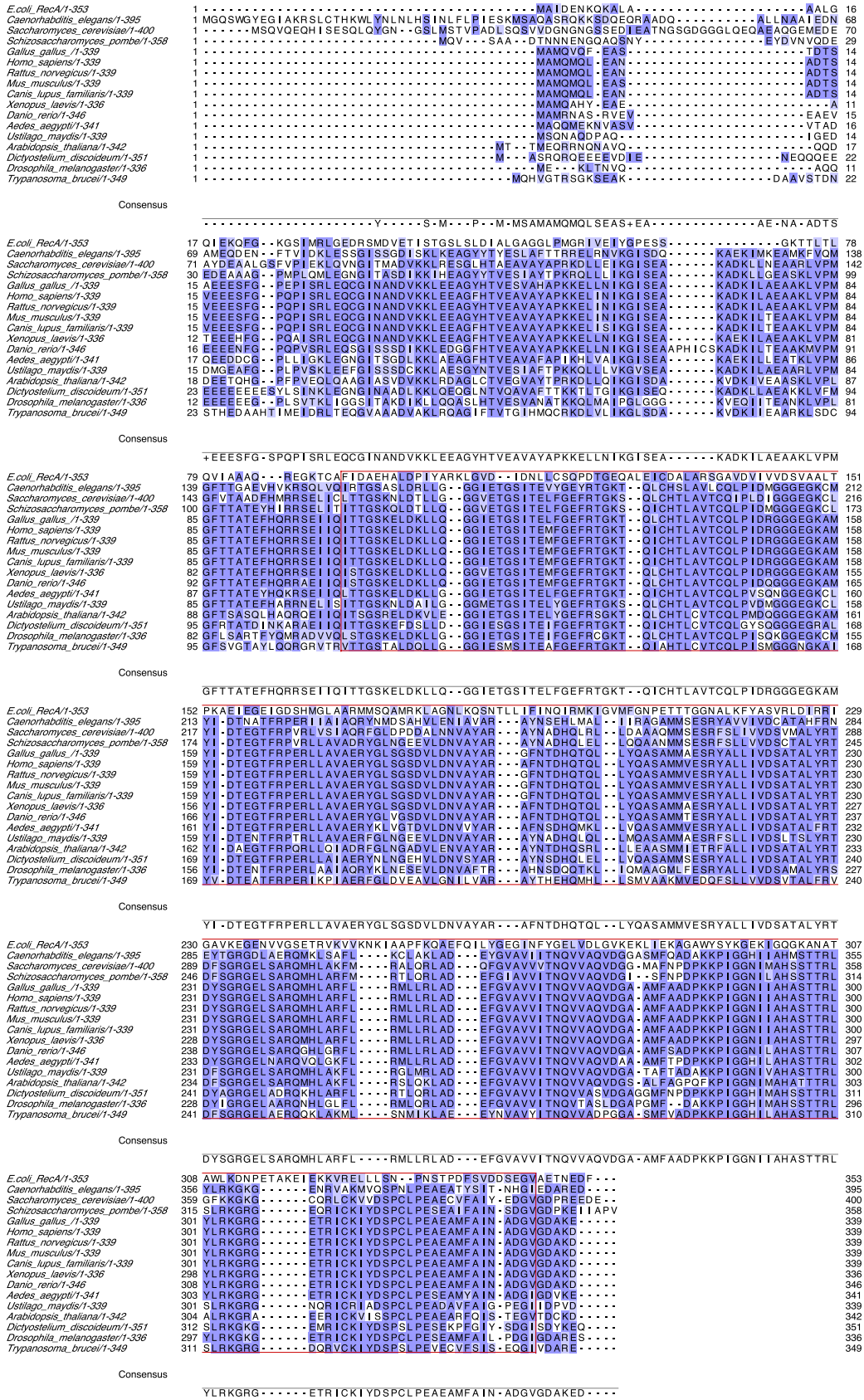

Figure S1 : Rad51 is highly conserved throughout eukaryotes. Rad51 protein sequences of different organisms were obtained from NCBI protein database (http://www.ncbi.nlm.nih.gov/protein/) and DictyBase (http://dictybase.org/). (A) Alignment of Rad51 orthologs from 16 species are shown, along with bacterial RecA of *E.coli*. The sequences were aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/)[165]. The alignment file was edited using Jalview (http://www.jalview.org/)[166]. Dark blue colored amino acids indicate identity and light blue similarity. A consensus sequence derived from the conservation profile of the proteins aligned, is shown at the bottom. The area marked by a red border highlights the Rad51_Dmc1_RadA catalytic domain in the eukaryotic proteins.

Organism	Identity
E.coli	26.51%
X. laevis	72%
A.aegipti	71%
H.sapiens	74%
C.elegans	67%
D.melanogaster	65%
C.lupus familiaris	70%
S.pombe	71%
U.maydis	65%
A.thaliana	59%
T.brucei	52%
M.musculus	73%
G.gallus	70%
D.rerio	69%
S.cerevisiae	61%

Table S1: Percentage identity of the Dictyostelium Rad51 compared to that of other organisms. *Dictyostelium* Rad51 was subject to pairwise alignment with all the proteins used for the alignment in figure S1A.

Figure S2: The BRC repeats in *Dictyostelium*. The three BRC repeats were aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/)[165]. The alignment file was edited using Jalview (http://www.jalview.org/)[166].

Chapter 8: Bibliography

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