

Screening for inhibitors of and novel proteins within the homologous recombination DNA repair pathway

Submitted by Mr Guy L Kingham of Linacre College
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Supervised by Professor Thomas Helleday
The Gray Institute for Radiation Oncology and Biology
University of Oxford



Abstract

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The homologous recombination (HR) pathway of DNA repair is essential for the faithful repair of double-stranded DNA breaks (DSBs) in all organisms and as such helps maintain genomic stability. Furthermore, HR is instrumental in the cellular response to exogenous DNA damaging agents such as those used in the clinic for chemo- and radiotherapy. HR in humans is a complex, incompletely understood process involving numerous stages and diverse biochemical activities. Advancing our knowledge of the HR pathway in humans aids the understanding of how chemo- and radiotherapies act and may be used to develop novel therapeutic strategies. Recent studies have identified inhibition of HR as one of the mechanisms via which a number of recently developed chemotherapeutics have their effect. Accordingly, the clinical potential of HR inhibitors is under investigation. My work has centred around the identification of both novel HR proteins and novel, small molecule HR inhibitors. To further these aims, I have successfully employed high-throughput RNAi and small molecule screening strategies. RNAi screens are commonly used to identify genes involved in a given cellular process via genetic loss of function, whilst small molecule, cell based screens are a powerful tool in the drug discovery process.

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Abbreviations

17-AAG	17-N-Allylamino-17-demethoxygeldanamycin
53BP1	p53 binding protein 1
A11	PARP knockout MEF
A19	Wild type MEF
ADP	Adenosine diphosphate
alt-NHEJ	Alternative non-homologous end-joining pathway
ATCC	American Type Culture Collection
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3 related
ATRIP	ATR interacting protein
BACH1	BTB and CNC homology 1
BCR-ABL	Breakpoint cluster region Abelson
BER	Base excision repair
BIR	Break induced replication
BLM	Bloom's syndrome helicase
BRCA1	Breast cancer 1 early onset
BRCA2	Breast cancer 2 early onset
BRD1	Bromo-domain containing 1
BSA	Bovine serum albumin
c-Abl	c-Abelson tyrosine kinase

c9orf102	chromosome 9 openreading frame 102
CDC25	Cell division cycle 25
CDK	Cyclin dependent kinase
CHD8	Chromodomain helicase 8
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
CK2	Casein kinase 2
cKIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
CPT	Camptothecin
CTIP	CTBP interacting protein
DAPI	4',6-diamidino-2-phenylindole
DF	DharmaFECT
DMEM	Dulbecco's modified Eagle's minimum essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-dependent protein kinase, catalytic subunit
DNA2	DNA replication helicase 2 homologue
DR-GFP	Direct-repeat green fluorescent protein
DSB	Deoxyribonucleic acid double-strand break
DSBR	Double strand break repair
dsRNA	Double-stranded ribonucleic acid

DSS1	Deleted in Split hand/Split foot protein 1 homologue
EDTA	Ethylenediaminetetraacetic acid
EME1	Essential meiotic endonuclease I homologue
ERCC1	Excision repair cross-complementing rodent repair deficiency, complementation group 1
esiRNA	endoribonuclease prepared siRNA
EST	Expressed sequence tag
Exo1	Exonuclease 1
FA	Fanconi anaemia
FACS	Fluorescence activated cell sorting
FANCX	Fanconi anaemia complementation group <i>X</i>
FEN1	Flap endonuclease 1
GC	Gene conversion
GEN1	Gen homologue 1
GFP	Green fluorescent protein
HAT	Histone acetyl transferase
HBO1	lysine histone acetylase 7
HDAC	Histone deacetylase
HDAC10	Histone deacetylase 10
HDAC9	Histone deacetylase 9
HER2	Human epidermal growth factor receptor 2
HERC2	Hect domain and RLD 2
His	Histidine

HP1	Chromobox homologue 1
HR	Homologous recombination
HSP90	Heat shock protein 90
HUS1	Hus1 checkpoint homologue
ICL	DNA inter-strand crosslink
IR	Ionising radiation
JMJD1B	Jumonji domain containing
KAP1	Kinesin-ii-associated protein
LIGIV	LigaseIV
MDC1	Mediator of DNA-damage checkpoint 1
MEF	Mouse embryonic fibroblast
MK2	mitogen-activated protein kinase-activated protein kinase 2
MLH1	MutL homolog 1
MMC	Mitomycin C
MRE11	Meiotic recombination endonuclease 11 homologue
mRNA	Messenger ribonucleic acid
MUS81	Mus81 endonuclease homologue
NADH	Nicotinamide adenine dinucleotide
NBS1	Nijmegen breakage syndrome 1
NCI	National Cancer Institute
NER	Nucleotide excision repair
NHEJ	Non-homologous end-joining

NT	Non-targeting
p38MAPK	p38 mitogen-activated protein kinase
p53	Tumour suppressor protein 53
PALB2	Partner and localiser of BRCA2
PARP	Poly(adenine diphosphate-ribose) polymerase
PARP1	Poly(adenine diphosphate-ribose) polymerase 1
PBS	Phosphate buffered saline
PCI	PCI-24781 HDAC inhibitor
PCNA	Proliferating cell nuclear antigen
PDGFR	Platelet derived growth factor receptor
PFA	Paraformaldehyde
PHD	Plant homeo domain
PI3K	Phosphoinositide 3-kinase
PIAS1	Protein inhibitor of activated STAT protein 1
PIAS4	Protein inhibitor of activated STAT protein 4
PIKK	Phosphatidyl inositolkinase related kinase
PNK	Polynucleotide kinase
POL ζ	DNA polymerase zeta
POL λ	DNA polymerase lambda
POL μ	DNA polymerase mu
POLX	Polymerase X family
qPCR	quantitative real-time polymerase chain reaction
RAD1	Radiation sensitivity abnormal 1

RAD26L	Radiation sensitivity abnormal 26 like protein
RAD50	Radiation sensitivity abnormal 50
RAD51	Radiation sensitivity abnormal 51
RAD51AP1	RAD51 associated protein 1
RAD51B	Radiation sensitivity abnormal 51B
RAD51C	Radiation sensitivity abnormal 51C
RAD51D	Radiation sensitivity abnormal 51D
RAD52	Radiation sensitivity abnormal 52
RAD54	Radiation sensitivity abnormal 54
RAD9	Radiation sensitivity abnormal 9
RAP80	Receptor associated protein 80
RFC	Replication factor C
RIDDLE	Radiosensitivity, immunodeficiency, dysmorphic features and learning difficulties
RISC	Ribonucleic acid-induced silencing complex
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RNF168	Ring finger protein 168
RNF8	Ring finger protein 8
RPA	Replication protein A
rpm	Revolutions per minute
RT-PCR	Real-time polymerase chain reaction
RuvA	Resolvasome subunit A

RuvB	Resolvasome subunit B
RuvC	Resolvasome subunit C
SDSA	Synthesis dependent strand annealing
shRNA	Short hairpin ribonucleic acid
siRNA	Small interfering ribonucleic acid
SLX1	Structure specific endonuclease 1
SLX4	Structure specific endonuclease 4
SSA	Single strand annealing
SSB	Deoxyribonucleic acid single-strand break
SSBP	Single-stranded binding protein
ssDNA	Single-stranded deoxyribonucleic acid
SUMO	Small ubiquitin-like modifier 1
SIRT6	Sirtuin 6
TAO kinases	Thousand and one amino acid kinases
TBS	Tris-buffered saline
TET3	Tet oncogene family member 3
TOPBP1	Topoisomerase binding protein 1
TOPO3A	Topoisomerase III alpha
WRN	Werner's syndrome helicase/exonuclease
XLF	XRCC4-like factor
XPF	xeroderma pigmentosum, complementation group F
XRCC1	X-ray repair complementing defective repair 1
XRCC2	X-ray repair complementing defective repair 2

XRCC3	X-ray repair complementing defective repair 3
XRCC4	X-ray repair complementing defective repair 4
γ -H2AX	Phosphorylated histone H2AX

Chapter 1

Introduction

1.1 DNA damage and its repair

DNA within all cells is subject to constant damaging processes threatening to compromise the genetic stability of the host organism. Direct damage to individual nucleotides may arise from metabolic processes within the cell that generate reactive oxygen species or reactive compounds capable of forming DNA adducts. Alternatively, perturbation of DNA replication can lead to single or double stranded DNA breaks (SSBs or DSBs). Nucleotide damage, replication stress and direct induction of SSBs and DSBs may also form as a result of exposure to exogenous agents including UV light, ionising radiation (IR), tobacco smoke and numerous chemotherapeutics [197]. Whilst all lesions may be mutagenic, those leading to breaks in the phosphodiester backbone of DNA are the most serious and are potentially toxic to the cell. Repair of damaged DNA is, therefore, not just essential for maintenance of

the genetic code but also for cellular survival.

Responding to the spectrum of DNA lesions generated in the cell are a host of specialised DNA repair pathways conserved from bacteria to humans that vary in complexity from enzymatic mechanisms that directly reverse simple base modifications to elaborate, tightly regulated biochemical responses to complex damage. My work is primarily concerned with the repair, in human cells, of potentially cytotoxic DSBs.

In order to mount a response to a DSB, the cell must first identify damage and initiate a signaling cascade. These DNA damage dependent signaling events will culminate in the appropriate cellular responses which include not only repair of the damage but activation of cell cycle checkpoints, and modulation of the transcriptional and replication machinery. The first event of the DNA damage response is the direct recognition of the DSB and immediate chromatin modification. Two protein kinases have been shown to play a redundant signaling role at this stage - ataxia-telangiectasia mutated kinase (ATM) and DNA-dependent protein kinase (DNA-PK), the catalytic subunit of which is referred to as DNA-PKcs [189] (see below for further detail). The immediate target of these phosphatidylinositol 3-kinase (PI3K) related kinases (PIKKs) is the histone variant H2AX, which is phosphorylated within minutes of damage induction at serine 139 to produce phospho-H2AX (γ H2AX) [170], which now serves as a platform for both the accumulation of repair factors and the transmission of damage signaling. The damage recognition and initial signaling processes must all occur within the context of

the chromatin surrounding the DSB. The assembly of repair and signaling mediator proteins at such a site is ordered, rapid and hierarchical [12].

DSBs may result from multiple processes: replication across a SSB, colocalisation of two SSBs, enzymatic activity and physical or mechanical stress [113]. It is of paramount importance that cells repair DSBs, ideally without loss of genetic content, before cell division. This repair may broadly be grouped into two types, (i) direct ligation of DNA ends and (ii) homology directed repair. Direct ligation is simpler, occurs throughout the cell cycle and may result in the loss of genetic information. Homology directed repair is more complex, can only occur in S and G2 phases of the cell cycle due to the requirement of homologous DNA provided by the sister chromatid and is high-fidelity. An overview of the signaling and repair events following damage recognition in humans is included below with a focus on homology directed repair.

1.1.1 Damage recognition and signaling in the chromatin context

The chromatin, within which DNA is complexed, not only serves as a natural barrier to any DNA manipulation, but provides both a physical platform for recruitment of repair factors as well as a supportive environment for damage signaling and signal amplification. Multiple, sequential post-translational modifications converge on the chromatin surrounding a DSB and serve to both enable and regulate repair.

Central to the recognition and damage signaling process are the three PIKKs (Fig 1.1), namely the ATM and ATM- and Rad3-related (ATR) kinases and DNA-PKcs. Recruitment and subsequent activation of these PIKKs to sites of DNA damage in all cases is mediated by an evolutionarily conserved motif [46]. Both ATM and DNA-PKcs, along with the MRN complex (described below) are the primary recognition factors for, and are activated by, double stranded DNA breaks [194, 60, 111]. In the case of DNA-PKcs, this recognition is mediated by its binding partner, the DSB binding Ku70/80 heterodimer (see below for further details). In contrast to both ATM and DNA-PKcs, which bind to DSBs [194, 60], ATR (in complex with its interaction partner, ATRIP) responds to the accumulation of single-stranded DNA (ssDNA) [233] following either replication stress or DNA resection at the DSB. This ssDNA is quickly complexed by the ssDNA binding protein RPA forming the ssDNA:RPA complex that activates ATR (see below for further details).

ATM has been shown to facilitate DSB repair in regions of compacted (hetero-)chromatin [38] via phosphorylation of proteins required for heterochromatin formation, such as KAP-1 and HP1[58, 38]. The assembly of repair proteins is also activated by phosphorylation. ATM and DNA-PKcs generate γ H2AX, which recruits the mediator protein MDC1 [192], itself constitutively phosphorylated by Casein Kinase 2 (CK2). Phospho-MDC1 may now bind those components required for further damage signaling and break site processing; RNF8 and NBS1 (part of the MRN complex essen-

tial for initiating homologous recombination or HR, see below) respectively [185, 132, 103] - see below for further details.

Further damage induced post-translational modifications are not limited to phosphorylation. Though a spectrum of modifications are generated in response to DNA damage, of particular recent interest is the ligation of ubiquitin and small ubiquitin like modifier (SUMO) proteins to the chromatin surrounding the break site as well as the poly-ADP-ribosylation catalysed by poly-ADP-ribose polymerase 1 (PARP1).

Ubiquitination has a well defined role in the labeling of proteins for degradation via the 26S proteasome, in addition, either mono- or poly-ubiquitination/SUMOylation may serve as signaling and/or recruitment moieties. Both modifications result from multi-step processes requiring the sequential action of activating (E1), conjugating (E2) and ligase (E3) activities with the E2 and E3 enzymes conferring substrate specificity upon the reaction [73].

Recent studies have illuminated the importance of the E3 ubiquitin ligases RNF8 and RNF168 and the RNF8 interaction partner HERC2 in the early stages of repair foci formation [83, 103, 127, 222, 188]. RNF8 is one of the first proteins recruited to γ H2AX via phospho-MDC1, where it interacts with HERC2. The RNF8-HERC2 complex guides RNF8 to its E2 conjugation partner UBC13 [11]. The resultant histone H2A and H2AX poly-ubiquitination surrounding the break site facilitates recruitment of RNF168, which also partners with UBC13 to raise H2A and H2AX ubiquitination levels

to an appropriate level to recruit the downstream repair proteins 53BP1 and BRCA1 (via its ubiquitin binding interaction partner RAP80) [41, 188]. The physiological relevance of post damage histone ubiquitination is emphasised by studies showing RNF168 mutation in a patient with the radiosensitising RIDDLE syndrome [188]. The roles of BRCA1 ubiquitination activity have been further expanded with the proposal that BRCA1 mediated ubiquitination of histone H2A *in vivo* protects genome integrity via the maintenance of heterochromatin structure [232] (DNA in its highly compacted heterochromatic form is not accessible to DNA repair factors [58]). This finding provides an extra layer of mechanistic understanding to the well documented tumour suppressive role of BRCA1.

In parallel to ubiquitination, SUMOylation (ligation of the small ubiquitin like modifying protein to a given target) following DNA damage is also instrumental to a successful cellular response to DSBs [230]. The SUMO ligases PIAS 1 and PIAS 4, similarly to RNF8 and RNF168, are required for recruitment and SUMOylation of 53BP1 and BRCA1 to sites of DSBs, where these repair factors are themselves SUMOylated [143, 54]. Moreover, it appears that SUMOylation of histones H2A and H2AX by PIAS1 and PIAS4 is required upstream of RNF8/RNF168 ubiquitylation in the repair process [54].

The theme of enzymatic polymerisation surrounding the break site is further exemplified by the action of PARP1. This multifaceted enzyme competes with the KU70/80 heterodimer for the binding of free DNA ends at

the break site, serving both to promote the DNA damage response via ATM activation as well as HR repair by mediating the recruitment of the MRN complex [74, 63, 64]. The clinical relevance of PARP1 and its role in DNA repair is addressed at the end of this chapter.

The concerted action of the ubiquitin and SUMO ligases as well as PARP1, together with other post-translational histone modifications in the region of a DSB, culminates with the recruitment of SUMOylated BRCA1 to a DSB (via its ubiquitin binding interaction partner RAP80), as well as recruitment of SUMOylated 53BP1, through less clear mechanisms [54]. These repair factors, together with the MRN complex recruited via phospho-MDC1, enable the initiation of HR repair.

1.1.2 DNA damage and cell cycle checkpoints

The initial recognition of DNA damage, as detailed above, serves not only to recruit and activate the repair machinery but to initiate a diverse and essential series of events within the cell to limit the effects of the damage and ensure the health and genetic integrity of the organism.

Sensing and initial signaling of DNA damage is performed chiefly by the DNA-PKcs, ATM and ATR kinases. For DSBs such as those induced by IR, the MRN complex, which is required for processing of the break site in HR (see below) recruits and activates ATM [111]. In the case of ATR, as well as its role in signaling following resection (outlined below), it is also activated at sites of replication stress. In such situations, the continued action

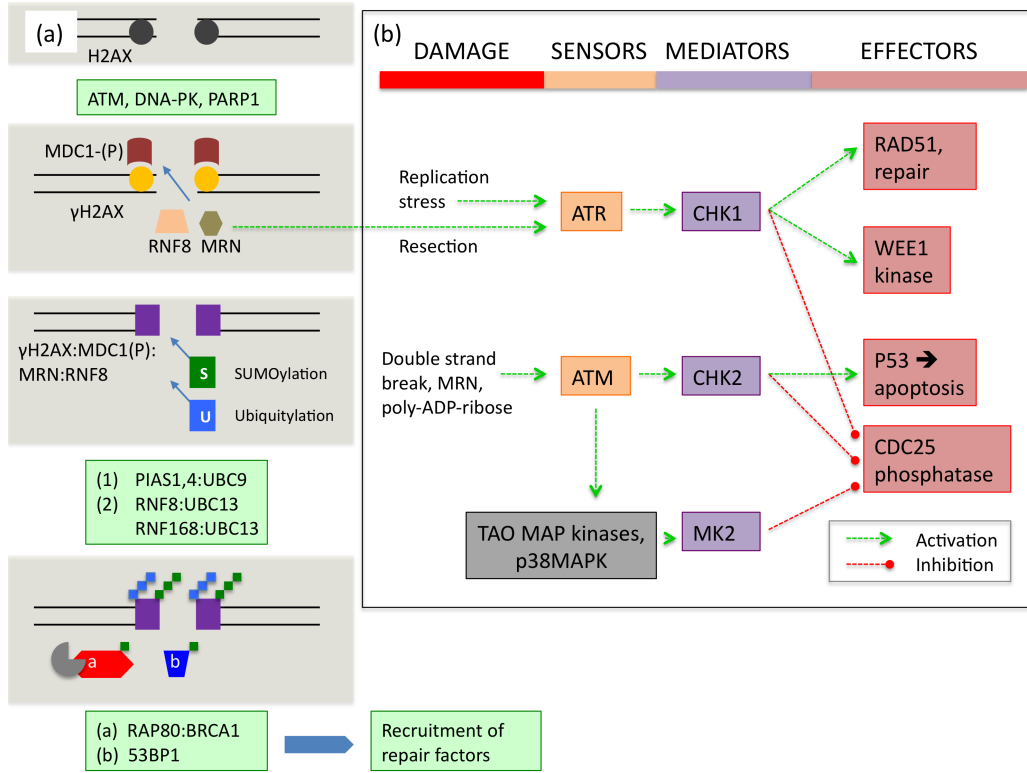


Figure 1.1: Overview of the initial events following DSB induction. **(a)** Damage is recognised by either ATM or DNA-PKcs leading to phosphorylation of histone H2AX to yield γ H2AX. PARP1 also recognises damage at this stage. Binding of phospho-MDC1 provides a docking site for the MRN complex and RNF8. A series of recruitment events leads to histone H2A modification by SUMO and ubiquitin ligases of the PIAS and RNF families, respectively, culminating in the recruitment of SUMOylated BRCA1 and 53BP1. **(b)** Following damage, the sensor kinases ATM and ATR are activated which, via mediator kinases, lead to activation of damage effectors exerting control over the cell cycle, apoptosis and DNA repair activation.

of the MCM replicative helicase following replication fork stalling reveals regions of ssDNA, which serve to recruit the ssDNA binding protein RPA. The RPA coated ssDNA provides a platform for both the recruitment ATR and its catalysis of the phosphorylation of the RAD17-RFC clamp loader. The phosphorylated RAD17-RFC clamp loader is then responsible for loading of the DNA damage specific 9-1-1 (RAD9-RAD1-HUS1) clamp complex and subsequent TOPBP1 binding. The assembly of these signaling components in response to replication stress further activates ATR kinase activity and downstream checkpoint activation [69].

With damage recognition signaling initiated, be it for a DSB or stalled replication fork, signal amplification and downstream effects are controlled by the checkpoint kinases CHK1 and CHK2, which are primarily activated by ATR and ATM, respectively. Once activated, CHK1 is free to diffuse away from the chromatin sites of DNA damage [152] driving events throughout the cell. One of the primary roles of CHK1 and CHK2 is in halting the cell cycle following DNA damage. Their key targets to effect this response are the CDC25 group of phosphatases [176, 47] required for activating CDK2 and CDK1 and driving cells through the G1-S and G2-M stages of the cell cycle respectively [42, 186]. CDC25 phosphorylation targets these CDK regulators for swift destruction via the 26S proteasome, thus increasing the levels of CDK1 and CDK2 in their inactive, phosphorylated state. This cell cycle inhibitory action is further reinforced by the CHK1 mediated phosphorylation and activation of the WEE1 CDK targeting kinase, reviewed in [32].

Cell proliferation in the presence of DNA damage is also countered by the activation of p53 mediated apoptosis through CHK2 [231].

Recent work [169] has established that CHK1 and CHK2 are not the only effector kinases of the DNA damage checkpoint. The p38MAPK signaling axis, well characterised as a general cellular stress response, is also activated following DNA damage [167] via phosphorylation of the TAO MAP kinase kinase kinase (MAPKKK) directly by ATM. These events culminate in inactivation of CDC25C, mirroring the CHK1/2 dependent pathway [134]. Further work has established that in the absence of p53, the kinase directly responsible for cell cycle arrest in S phase via ATM/ATR driven CDC25 inactivation, is the p38MAPK binding partner, MK2 [169].

With the downstream targets of the ATM/ATR dependent checkpoint signaling pathway delineated into three separate pathways, it is also important to appreciate that CHK1 directly influences the repair machinery. Prominent such examples include CHK1 phosphorylation of RAD51 to promote HR [184] and the control of crosslink repair through CHK1 phosphorylation of FANCD2 [164]. CHK1 also serves as part of the replication stress checkpoint by regulating replication origin firing [49]. It has been demonstrated that there is an enrichment of the initiation of late DNA replication origins in CHK1 inhibitor (UCN-01) treated cells following replication stress [49] and it is proposed that CHK1 acts to arrest the normal origin firing programme in cells undergoing replication stress.

The relevance of the DNA damage response is best appreciated when con-

sidering its physiological role as an anticancer barrier [8]. This role has been brought to light upon the consideration of two key observations: firstly induction of the DNA damage response in tumour cell lines with overexpressed oncogenes [205] and secondly the induction of the DNA damage response in tumour cells isolated from patients in a preadjuvant setting [39]. Based upon these findings, a model has been proposed [8] in which activated oncogenes generate replication stress, activating the ATM and ATR signaling pathways and channelling a response to the master tumour suppressor p53 driving cell cycle arrest, senescence [9, 59, 10, 23], or apoptosis [27]. In this manner, cells that are prone to genomic instability are removed from the dividing population, protecting the organism from tumourigenesis. This model is broadly supported when one considers the prevalence of mutations in key DNA damage response proteins in cancer patients (Table 1.1).

1.1.3 Direct ligation of double strand breaks - end joining

The classical non-homologous end joining (NHEJ) pathway is predominantly responsible for DSB repair in the absence of homologous, undamaged DNA to act as a repair template i.e. in the G1 phase of the cell cycle. Although there is growing evidence for alternative end joining pathways, these are outside the scope of this brief overview. Successful, classical end joining requires five distinct biochemical functions, namely:

1. Recognition, binding and tethering of free DNA ends.

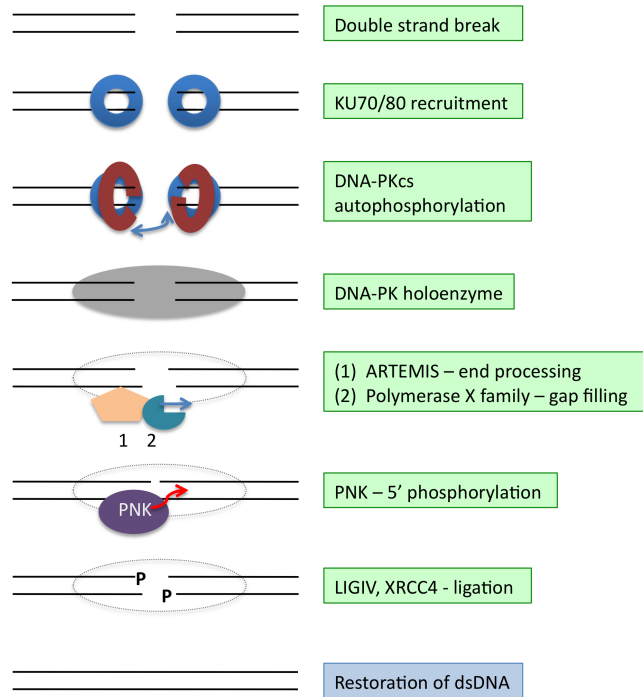


Figure 1.2: Non-homologous end joining: Following induction of a double stranded DNA break (DSB) and initial DSB recognition by the Ku70/80 heterodimer, DNA-PKcs is recruited whereupon it autophosphorylates. The KU70/80:DNA-PKcs holoenzyme (DNA-PK) then provides the platform for end processing by the Artemis nuclease, members of the POLX polymerase family and the PNK kinase/phosphatase. Following end processing, restoration of dsDNA is mediated by LigaseIV and XRCC4.

2. Signaling of damage status and recruitment of repair proteins.
3. Nucleolytic processing and cleaning of irregular ends.
4. DNA polymerase filling of DNA gaps.
5. Ligation of clean ends.

The NHEJ pathway (Fig 1.2) contains members responsible for each of these functions and is initiated by the recognition of the free DNA ends of a DSB by the highly abundant KU70/80 heterodimer (KU), which binds tightly to DNA ends[113]. The toroidal structure of KU, as revealed by X-ray crystallography [211], rationalises its ability to bind dsDNA and facilitate recruitment of other NHEJ pathway members. These may be recruited to the KU:DNA complex in any order [122], but will be dealt with here in the order outlined in the above list.

The signaling component of NHEJ is provided by the largest kinase in biology, DNA-PKcs. With analogy to Ku, this 469kDa kinase forms a gapped ring structure [181], which is likely to tether the two ends of a DSB. DNA-PKcs forms a complex with Ku:DNA, which together is referred to as DNA-PK. The kinase activity of DNA-PKcs is activated upon binding to dsDNA ends [66] and is required for NHEJ activity [107]. Upon activation, DNA-PKcs autophosphorylates more than 16 sites. Though the roles of all these phosphorylation events are incompletely understood, some of this activity is believed to be involved in promoting disassembly of the DNA-PK holoenzyme following damage repair [126].

Once the free DNA ends have been tethered, they may require nucleolytic processing to remove nucleotide overhangs and damaged bases. The Artemis nuclease interacts with DNA-PKcs and fulfills this role by virtue of its multiple activities, including 5'- and 3'- endonucleolytic and 5'-exonucleolytic activities [113]. The blunt DNA ends are now amenable to further processing by the dual function polynucleotidekinase (PNK), which prepares DNA ends for ligation by exploiting both its 5' kinase and 3' phosphatase activities. PNK has also been shown to interact with phosphorylated XRCC4, part of the ligase machinery [101].

Artemis activity at the DSB may lead to 3' or 5' DNA overhangs, these require filling by specialised DNA polymerases of the POL X family (POL μ , POL λ). The nature of the damage site necessitates the unique polymerase characteristics displayed by POL μ and POL λ , such as synthesis using dNTPs or rNTPs, the ability to synthesise across a discontinuous gap and template-independent synthesis [113].

Finally, with the DNA ends prepared for ligation, a complex including DNA ligase IV restores the structural integrity of the damaged DNA. Ligase IV is required for the ligation reaction and is found in complex with XRCC4, which acts to stabilise it and stimulate its activity. Similar to XRCC4, which has no known enzymatic activity, is XLF. Along with XRCC4, XLF stimulates ligase IV activity - both these ligation accessory proteins are required for NHEJ [126].

1.1.4 Homology directed repair of double strand breaks - homologous recombination

Similarly to NHEJ, a number of essential steps are required for homology directed repair of a double strand break by HR. Successful repair requires a repair template, provided by the non-damaged sister chromatid. Accordingly, HR can only occur within the S and G2 phases of the cell cycle. Aside from this general requirement, HR requires 3'-overhanging ssDNA generated by resection, recombinase activity, DNA synthesis and repair structure resolution factors for completion. HR is necessarily more complex than NHEJ and this fact is reflected at each stage of the repair process:

1. DNA end processing and 5'-3' resection.
2. Joint molecule formation and repair synthesis.
3. Repair structure dissolution or resolution.

DNA end processing and 5'-3' resection

With analogy to the damage recognition process outlined above, resection at the double strand break site is a stepwise, sequential process. Resected DNA ends (3' ssDNA overhangs) become coated by RPA and this complex serves as both a substrate for HR and as an HR signaling platform via activation of ATR and its downstream target kinase CHK1 [90]. Resected DNA is, therefore, a prerequisite for HR.

The initial steps in resection are performed by the multi-component MRN (MRE11-RAD50-NBS1) complex (Fig 1.3), whose roles in damage recognition have been introduced above. *In vivo*, MRE11 forms a MRE11₂RAD50₂ core complex to which two monomers of NBS1 bind via interactions with MRE11 [109]. Whilst it must be borne in mind that MRN functions as a complex, it is informative to distill it into its constitutive components for individual consideration. The MRE11 nuclease can bind to and synapse DNA ends [217], whilst its endo- and exonuclease activities are believed to be responsible for initial processing of the break site [109]. It is important to note, however, that the requisite 5'-3' exonuclease activity for initiation of HR has not been detected in MRE11, leaving an essential role for further nucleases to play, such as EXO1 and DNA2, discussed below. RAD50 provides a structural role within the complex, with an ATP binding cassette coupled to an extensive coiled coil domain facilitating tethering of two DNA molecules [35], for example damaged DNA and a repair template [109]. The Walker A and Walker B boxes reside at the N- and C-terminals of this extended protein, but form a bi-partite ATPase that is capable of limited dsDNA terminus unwinding [76]. NBS1 has been described above to localise the MRN complex to γ H2AX via interaction with phospho-MDC1 and also stimulates the activity of both MRE11 and RAD50 [160]. The key physiological role of these (and other) repair factors in maintaining genomic stability is underscored by the observation that mutation of MRE11, NBS1 and RAD50 have all been associated with predisposition to cancer (as well as other phenotypes)

in ataxia-telangiectasia like disease (A-TLD), Nijmegen Breakage Syndrome (NBS) and an NBS-like condition, respectively [187, 212, 207]. The role in human disease of numerous proteins from almost the whole spectrum of DNA repair pathways is highlighted in Table 1.1 at the end of this Chapter.

The MRN complex senses (via the MRE11 dimer and its DNA end bridging activity [217]) and rapidly localises to DSBs [137] where it is also required for ATM activation [20]. With MRN in place and initial processing of the break complete, resection may now proceed, to which end the CTIP protein is recruited. Structural studies with the fission yeast CTIP homologue (Ctp) and yeast Nbs1 have clarified the mechanism of this recruitment, with Ctp tethered to a flexible arm of Nbs1 [216]. Once recruited to a DSB, the CTIP endonuclease complements the endonuclease activity of MRN and also leads to ssDNA generation, RPA recruitment and ATR activation [177]. It has recently been proposed from studies in fission yeast and chicken cells that CTIP, assisted by its interaction partner BRCA1, collaborates with the MRN complex to remove covalently trapped protein:DNA complexes at DSBs - exemplified by topoisomerases immobilised following topoisomerase poison treatments [70, 151]. Further development of this idea has been provided by work in *Schizosaccharomyces pombe* showing that both Mre11 nuclease activity and Ctp1 are required for the removal of the Ku70-80 heterodimer and the MRN complex from DSBs in order to facilitate RPA loading and successful resection [110].

With resection now initiated via MRN and CTIP, processive tracts of

3' overhanging ssDNA appropriate for HR must be generated. Consistent with the roles played by their homologues in *S.cerevisiae*, the cooperation of the Bloom's syndrome helicase (BLM) and EXO1 5'-3' exonuclease allows processive resection [61]. The role of such a helicase/nuclease partnership in generating ssDNA for HR is a conserved theme across evolution. It has recently been shown that there are multiple such activities in humans with the two exonucleases EXO1 and DNA2 playing central roles. In a series of illuminating *in vitro* studies, Nimonkar et al [153] reconstitute the resection machinery and highlight the separate roles of BLM-DNA2-RPA-MRN and EXO1-BLM-RPA-MRN complexes in long range (greater than 1kb) resection. In both complexes, the helicase activity of BLM is of central importance, with the helicase activity of the DNA2 dual function helicase-nuclease being dispensable. Both complexes are capable of processive resection. The EXO1 complex, in particular, requires the stimulation of nuclease activity through its interaction with MRN.

Recent work conducted in *S.cerevisiae* using covalently blocked DSB ends generated by the topoisomerase-like transesterase Spo11 has shed light upon the coordination of Mre11 and Exo1 in resection [55]. Exploiting both Mre11 and Exo1 mutant yeast strains, the authors propose a model in which Mre11 or Sae2 (*S.cerevisiae* CTIP homologue) endonuclease activity generated nicks flanking the ends of a DSB. These nicks serve as initiation sites for bidirectional resection by Exo1 and/or Sgs1-Dna2 away from the DSB (5'-3'), and by Mre11 towards the DSB end (3'-5'). This model is depicted in Fig 1.3

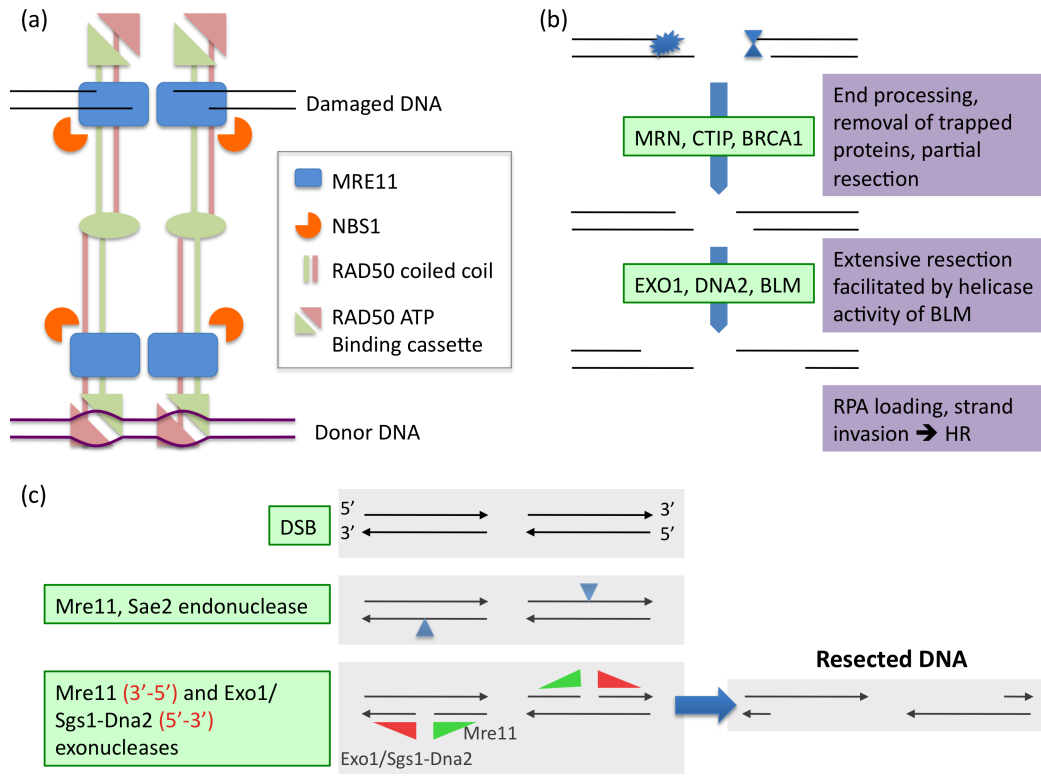


Figure 1.3: The MRN complex and resection **(a)** The MRN complex, comprising MRE11, RAD50 and NBS1. The MRE11 monomers bind at the DSB via interaction with NBS1. Donor DNA is tethered to the DSB via RAD50. The RAD50 ATP binding cassette binds to and catalyses limited dsDNA unwinding of the donor DNA. **(b)** Overview of resection with end processing followed by extensive resection providing a substrate for HR. **(c)** Resection is initiated (according to a recently proposed model in *S.cerevisiae* [55]) by Mre11 and Sae2 (*S.cerevisiae* CTIP homologue) endonuclease activity. Exonuclease activity then proceeds from this endonucleolytic nick mediated by Mre11 in the 3'-5' direction and by Exo1/Sgs1-Dna2 in the 5'-3' direction to reveal fully resected DNA at the DSB site. Sgs1 is the *S.cerevisiae* orthologue of the BLM helicase.

and whilst not yet demonstrated in human cells, provides a cell based model to relate to the *in vitro* studies described above [153].

It is critically important to regulate the generation of ssDNA downstream of CTIP recruitment to avoid inappropriate HR and this is achieved on multiple levels. Firstly, cell cycle dependent transcriptional control of CTIP expression, in part due to CTIP's ability to activate its own promoter via the E2F/RB pathway [115], ensures minimum levels during the G1 phase of the cell cycle [227]. CDK phosphorylation at the conserved Thr847 also controls the resection-promoting role of CTIP [84]. Further regulatory fine tuning, targeting to break sites and commitment to the HR pathway is achieved by the BRCA1 tumour suppressor, an E3 ubiquitin ligase commonly mutated in breast cancer [218, 228, 229]. This interaction is controlled by CDK-dependent CTIP phosphorylation. More recent work [95] has uncovered the role of the lysine deacetylase SIRT6 in activating CTIP in response to DSBs and thereby promoting resection and HR. This discovery reinforces the importance of resection regulation through CTIP and it seems likely that further players in this process await discovery and characterisation.

Joint molecule formation and repair synthesis

With 3' overhanging ssDNA revealed and stabilised by interaction with RPA, the path is cleared for the RAD51 recombinase to be loaded with the assistance of BRCA2 (see below and Fig 1.4) and direct the homologous pairing of resected DNA with the donor DNA that will provide the repair template.

RAD51 displaces RPA to polymerise around ssDNA to form a helical nucleoprotein filament in which the ssDNA exists in an extended conformation [13], aiding the search for and pairing with donor DNA. RAD51 is the sole mitotic recombinase in humans and is highly conserved from *E.coli* and throughout evolution [174]. RAD51 is a DNA-dependent ATPase [13] and although ATP hydrolysis is not required for recombination [144], it has been shown to play a role in dissociation of the RAD51:DNA complex following homologous pairing [24]. An overview of the strand invasion process is provided in Fig 1.4.

Though the strand invasion process requires only RAD51, there are five RAD51 paralogues (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3), which assist in RAD51 nucleofilament formation [225]. The paralogues form two distinct biochemical complexes (i) RAD51B, C, D and XRCC2 in the 'BCDX2' complex and (ii) RAD51C together with XRCC3. This biochemical distinction may be related to a proposed separation of roles for the paralogues downstream of nucleofilament formation [225]. The BCDX2 complex has affinity for branched DNA and DNA nicks whilst the RAD51C:XRCC3 complex has been associated with Holliday junction resolvase activity [174, 118].

The assembly of RAD51 onto resected DNA requires the concerted action of a number of factors, since RPA-coated ssDNA presents a physical barrier to RAD51 nucleofilament formation, which must be surmounted with the aid of mediators. In humans, this mediation is controlled by the tumour suppressor BRCA2, mutations in which (along with mutations in BRCA1, see above) lead to genomic instability and predisposition to breast, ovarian

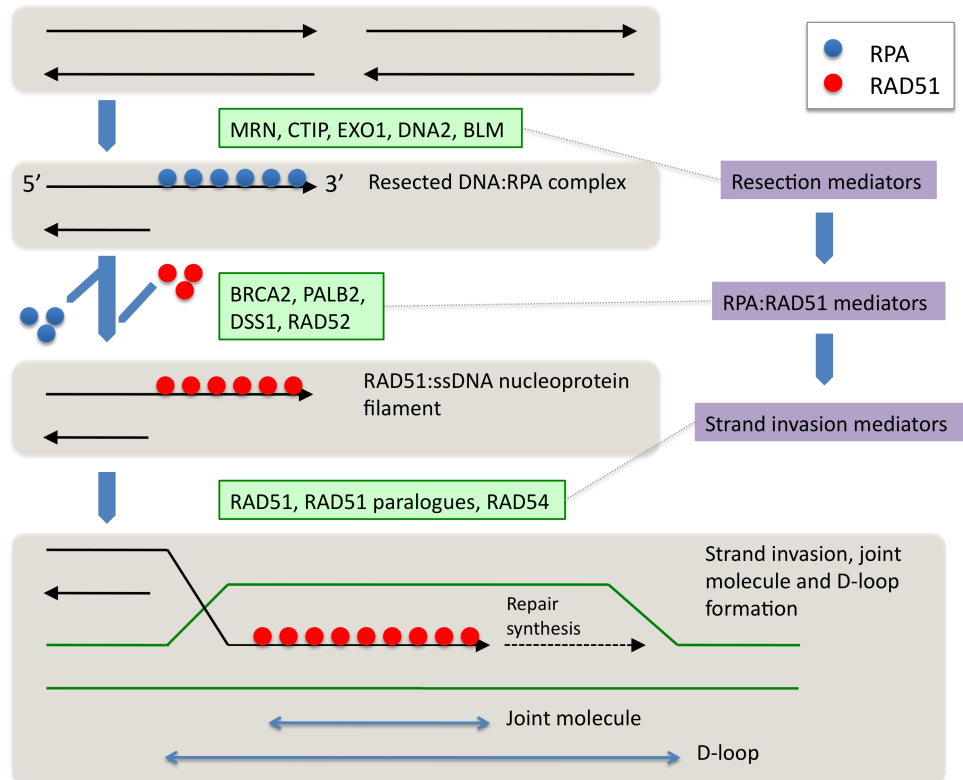


Figure 1.4: The core initiating steps of HR. Following resection, the RPA:ssDNA complex must be processed by removal of RPA and concomitant RAD51 loading to produce a ssDNA:RAD51 nucleoprotein filament, which then initiates strand invasion of the double stranded, homologous, donor DNA template. Mediators of each of these key processes are boxed in green. The strand invasion process results in both joint molecule and D-loop formation.

and other cancers [75]. Since the cloning and sequencing of this pre-eminent DNA repair gene [220, 203], it has been clear how important BRCA2, along with BRCA1, is in cancer development. It has taken some time and great effort to begin to understand the mechanisms by which BRCA2 facilitates HR.

Initial work on BRCA2 served to identify the presence, conservation and importance of multiple repeat domains (BRC domains) with RAD51 binding activity [16, 219], whilst yeast two-hybrid experiments verified this interaction [219]. Further work established the likely role of BRCA2 in HR with similar DNA repair defects in BRCA2 disrupted cell lines to RAD51 knock-out cells [29, 159, 142] and the demonstration of compromised RAD51 foci formation in response to DNA damage in BRCA2-truncated cells [226].

Notwithstanding the knowledge base already established on BRCA2 function, the greatest strides in defining the activity of BRCA2 in humans have come very recently with long anticipated purification of this giant 384kDa protein by three different groups using human and yeast expression systems [92, 204, 117]. These triumphs have given unprecedented biochemical insight into how BRCA2 interacts with RPA, DNA and RAD51 *in vitro* to promote strand exchange.

This wealth of information is collated and interpreted in a recent review [75], which provides a convenient framework for considering BRCA2 function. Crucially and unsurprisingly, given the level of previous knowledge, all of the studies support the role of BRCA2 in promoting strand exchange by RAD51.

The ability of BRCA2 to act as an HR mediator is also demonstrated, BRCA2 facilitates the interaction of RAD51 with the RPA:ssDNA complex [92, 117], although not necessarily by direct interaction with RPA. It is also proposed [92] that BRCA2 stabilises the RAD51:ssDNA filament by inhibiting RAD51 ATPase activity.

With BRCA2 now firmly established as an essential mediator of RAD51 dependent strand invasion, it is worth noting that this activity is not achieved in isolation. Various BRCA2 partners have been identified, which serve to stabilise and stimulate BRCA2 activity. Amongst the recent work using purified, full length BRCA2, it has been shown that DSS1, a known interaction partner of BRCA2 [128] stimulates the BRCA2 mediated binding of RAD51 to RPA:ssDNA [117]. Another BRCA2 binding partner, PALB2 both maintains the stability of BRCA2, directs its proper localisation within the nucleus and colocalises with BRCA2 to sites of DNA damage [223]. The importance of this particular interaction is demonstrated by the association of mutation of PALB2, a Fanconi anaemia gene, with familial cancers [45, 166]. A recent and intriguing addition to the the list of RAD51-interacting proteins is Cyclin D1, which directly binds RAD51 and is recruited to DSBs in a BRCA2 dependent manner [93], the precise mechanistic role of this interaction is yet to be uncovered but illustrates the, at times, surprising complexity of BRCA2-mediated RAD51 loading process.

Though it is clear that BRCA2 plays an irreplaceable role as a RAD51-RPA:ssDNA mediator, it is perhaps surprising that RAD52 (which serves

the mediator function in yeast, which lacks BRCA2) does not exhibit an equivalent role in humans. Recent work has, however, shone some light on the importance of RAD52 in the absence of BRCA2 [51]. The authors show that RAD52 is synthetic lethal with BRCA2 and that RAD52 colocalises with RAD51 at sites of DNA damage in a BRCA2 independent manner. The authors go on to suggest that RAD52 is required for HR in cells lacking functional BRCA2.

Through the coordinated action of BRCA2 and its interacting proteins, the 3' hydroxyl group of the resected DNA is placed via the RAD51 nucleoprotein filament in a position to prime repair synthesis using homologous DNA as a template. It is, however, unclear precisely which polymerases perform this task *in vivo* [174], although recent work has shown that a complex containing the trans-lesion synthesis polymerase POL ζ and REV1 is required for resistance to DSB inducing agents and that this protein complex is required for efficient HR [179]. The proposed role for POL ζ in HR is consistent with the identification of the POL ζ subunit REV3 as a candidate tumour suppressor with a significant reduction of Rev3 gene expression discovered in 74 colon carcinomas [18] .

The formation of the joint molecule structure, or synapsis followed by initiation of repair synthesis marks the successful initiation of HR. Further HR proteins are now required to complete repair in the post synaptic stages. Key activities at this point include branch migration of the junction formed by strand invasion as new DNA is synthesised, the remodelling of chromatin

surrounding the repair site and the resolution/dissolution of the repair structure to yield fully repaired double stranded DNA (see below).

One remarkably versatile protein reviewed in [130] is involved in nearly all of the processes outlined above. RAD54 is an ATP-dependent dsDNA translocase and chromatin remodeller of the SF2 family of helicases. It is capable of interacting with and stimulating RAD51, facilitating D-loop formation, promoting DNA synthesis and branch migration, as well as having roles in the removal of RAD51 from double stranded DNA post repair and stimulating the activity of the MUS81-EME1 nuclease that is involved in collapsing the repair structure. With proposed roles throughout HR, genetic data in yeast have pointed towards its importance downstream of RAD51 i.e. post-synapsis [199].

Similarly to RAD51, RAD54 has paralogues, both in yeast and mammals. Its paralogue in humans, RAD54B, exhibits similar biochemical activities such as interactions with RAD51, dsDNA dependent ATPase activity and the ability to promote D-loop formation by RAD51 [174]. Mouse knockout models have allowed the placing of RAD54B in HR, demonstrating that it only plays a role in the absence of RAD54 [215].

Through a succession of events catalysed and regulated by the key HR players RAD51 and BRCA2 and their partners, processive, template directed DNA repair is initiated. The final stages of HR are no less remarkable, or simple, and are outlined below.

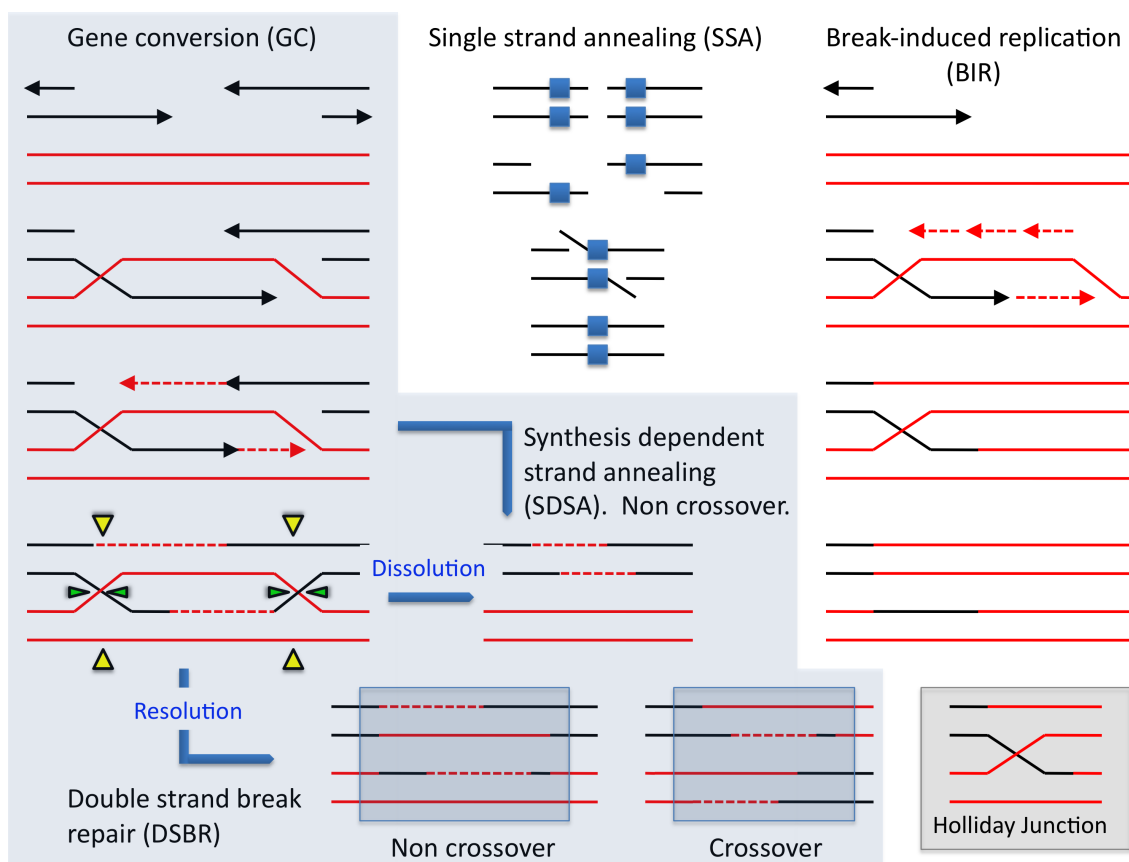
Repair structure dissolution or resolution.

There are numerous routes for repair to take following resection and joint molecule formation, these are illustrated in Fig 1.5. Briefly and with reference to [195, 174] and Fig 1.5, there are three core homology-directed pathways: gene conversion (GC), single strand annealing (SSA) and break-induced replication (BIR), which share common initiating stages but differ in their outcomes. All pathways are initiated by resection, as described above. A key intermediate in some of these pathways is the Holliday junction, formed following crossing over of the invading strand with the donor strand. The two dsDNA molecules of the repair process are now covalently joined, this structure must be resolved for repair completion (see below).

Gene conversion repairs two-ended DSBs and is subdivided into two pathways. In synthesis dependent strand annealing (SDSA), following D-loop formation and initial repair synthesis, the repaired strands can re-anneal. Although a Holliday junction is formed, the newly synthesised strands may re-anneal without resolvase activity and accordingly non-crossover products are formed. In the alternate mechanism (DSBR), D-loop migration facilitates the capture of the second resected end. Repair synthesis and ligation leads to double Holliday junction formation. The restoration of two dsDNA molecules now requires either the dissolution of the double Holliday junction by topoisomerase-mediated decatenation, specifically the partnership between the BLM helicase and TOPO3A [221], or its resolution by structure specific endonuclease activity. The nucleolytic resolution of the double Hol-

liday junction by structure specific endonucleases (see below) acting in the horizontal or vertical planes yields either crossover or non-crossover products. A far simpler repair pathway termed single strand annealing (SSA) may be used in regions of DNA containing repetitive sequence elements. In these cases, resection reveals intrachromosomal homology, which may be exploited as a repair template requiring no DNA synthesis. The pairing of the complementary strands revealed by resection leaves 3' ssDNA flap structures

Figure 1.5 (*following page*): **Gene conversion (GC)** - Following D-loop formation, the GC pathway diverges. In the absence of double Holliday junction formation, repair can continue via synthesis-dependent strand annealing (SDSA) to give non-crossover products. Alternatively, repair synthesis, D-loop migration and ligation can lead to double Holliday junction formation. The dissolution or resolution (by double strand break repair (DSBR)) of these structures leads to non-crossover (cleavage marked by blue arrows) and crossover products (cleavage marked by yellow arrows). **Single strand annealing (SSA)** - repeat sequences (shown in blue) facilitate local, intra chromosomal homology-driven pairing and generation of 3' ssDNA flaps, which are removed by nuclease activity. Ligation restores dsDNA with consequent loss of genetic information. **Break-induced replication (BIR)** - following D-loop formation, repair synthesis continues in two directions along the whole of the donor chromosome, Holliday junction resolution may be needed to finish repair.



which must be removed nucleolytically. SSA leads to loss of sequence information. The final repair mechanism that has been characterised occurs at single ended DSBs, such as those formed at collapsed replication forks and uses repair synthesis and D-loop migration along the whole of the undamaged chromosome to give a crossover product - this is termed break-induced replication (BIR).

As is now clear, HR produces numerous genetic outcomes. Aside from subpathway selection, the nature of restoring the joint molecule structure to its two constituent dsDNA molecules is the key to this variability. Since the proposal of the Holliday junction recombination intermediate in 1964, there has been the realisation that nuclease activity is required for the resolution of a number of proposed HR intermediates. Early studies identified enzymatic activities, or resolvases, capable of Holliday junction resolution in prokaryotes and T4 bacteriophage [139, 28, 88] with the prototypical resolvase characterised in *E.coli* and comprising three proteins: a motor (RuvB) and associated partner (RuvA) together with the RuvC nuclease, reviewed in [178]. The search for the eukaryotic equivalents of these model resolvases proved to be less immediately rewarding with no single candidate standing out. Perhaps due to the increased complexity of eukaryotic DSB repair there appears to be more flexibility and redundancy of endonuclease activity higher up the evolutionary tree. Aside from nucleases, there are likely to be a number of other necessary activities for Holliday junction resolution. Some intriguing candidates are the RAD51 paralogues RAD51C and XRCC3, cells deficient

in which having been demonstrated to exhibit reduced Holliday junction resolvase activity [118].

With reference to Schwartz *et al* [178], the spectrum of structure specific endonucleases involved in eukaryotic HR may be grouped into four superfamilies (i) XPF, (ii) MUS81/EME1, (iii) GEN1 and (iv) SLX1-SLX4. A great deal of characterisation work on these nucleases has been conducted in yeast, however, where possible I will focus on their human counterparts. With analogy to the bacterial RuvA,B,C machinery, the eukaryotic resolvases generally take the form of a nucleolytic and non-nucleolytic subunit.

The XPF superfamily is named after the XPF-ERCC1 complex. XPF is a member of the *Xeroderma pigmentosum* complementation group and is mutated in patients with this genetic condition, sensitising them to UV light and rendering them susceptible to cancers, especially of the skin [195]. Whilst not characterised as a Holliday junction resolvase, this complex is nonetheless important in DSB repair [4]. The *S.cerevisiae* orthologues of XPF-ERCC1 (Rad1-Rad10) remove non-homologous 3' flaps at DSBs by 5' endonucleolytic activity [157] and equivalent activity in XPF-ERCC1 has been deemed responsible for its role in facilitating HR in mammalian cells by SSA and GC [5].

The MUS81-EME1 complex is a further example of an endonuclease with multiple endonuclease activities required for HR, primarily at stalled replication forks and inter-strand crosslinks [67], that possesses 3'-flap and stalled fork structure endonuclease activities but not resolvase activity [26]. It is pos-

sible that the observed interaction and cooperativity of MUS81 with RAD54 [129] may target it to certain recombination structures.

The long quest for the mammalian resolvase was recently bolstered by the discovery of human GEN1 by mass spectrometry following the extensive fractionation of HeLa cell-free extracts and their screening for HJ resolvase activity [86]. GEN1 is a nuclease capable of a RuvC like symmetrical Holliday junction resolution as well as 5'-flap and model replication fork structure cleavage [86]. It seems unlikely, however, that this protein represents the sole human Holliday junction resolvase. Knockdown of GEN1 in human cells yields little phenotypic response to crosslinking agents, with a similar lack of phenotype demonstrable in yeast and *C.elegans* [178].

The final superfamily currently described is SLX1-SLX4, which was identified in *S.cerevisiae* by virtue of its synthetic lethality with the BLM orthologue Sgs1 [147]. The human SLX4 orthologue has recently been characterised by a number of groups [149, 50, 196]. Initial characterisation [149] demonstrated the interaction of SLX4 with, and its stimulatory activity upon, the structure specific endonuclease family members SLX1, XPF-ERCC1 and MUS81-EME1 together with cleaved branch structure binding activity. This study identified human SLX4 by virtue of its homology to its yeast counterpart. The authors demonstrated the interaction of endogenous SLX4 with SLX1 and suggest that it acts as a scaffold for SLX4 stimulated SLX1 dependent HJ resolvase activity [149]. This assertion of SLX4 providing a docking site for SLX1, XPF-ERCC1 and MUS81-EME1 nucleases has been

supported by studies showing that SLX4 interacts with SLX1 and XPF via yeast two-hybrid and immunoprecipitation assays and that SLX4-SLX1 complexes may be isolated from human cells and perform HJ resolution *in vitro* [50]. A third study identified SLX4 via different means [196] and solidifies the role of SLX4 and its binding partners as both HJ resolvases and structure-specific endonucleases capable of cleaving numerous DNA structures arising during replication fork stalling and HR. The authors [196] mined data from a proteomic screen using pulled down HA-tagged SLX4 complexes to identify its central position within different endonuclease complexes (identical to those already outlined above). The expanding role of SLX4 as a key component of the versatile nuclease toolkit within the cell is also beginning to come into focus with its function in interstrand crosslink repair being regulated by the Fanconi anaemia pathway (see below) and mutations of SLX4 now found in patients with Fanconi anaemia [190, 99]. All the above studies [196, 50, 149], though focused upon the identification of SLX4 containing complexes and their *in vitro* activity with respect to DNA structures arising during DSB repair and replication fork stalling, also demonstrate the broad cellular importance of SLX4 with its depletion sensitising human cells to a range DSB inducing agents.

It is increasingly evident that no single nuclease is required for HR in humans, however some recent work [214] goes some way to delineating the hierarchy of the competing Holliday junction resolution pathways. Using BLM (and therefore Holliday junction dissolution) deficient cells, the au-

thors demonstrate that depletion of either MUS81 and GEN1 or SLX4 and GEN1 leads to severe chromosomal aberrations i.e. impaired Holliday junction resolution [214]. The lack of such a phenotype in MUS81 and SLX4 depleted cells suggests GEN1 alone may be able to compensate for the other nucleases [214]. What is certain is that the spectrum of structure specific nucleases in humans work together, in multiple pathways to ensure proper repair, and that dysregulation of this activity can lead to severe consequences, such as Fanconi anaemia.

1.1.5 Crosslink repair and Fanconi anaemia

An additional DNA repair pathway that is both highly clinically relevant and intricately linked with HR is the Fanconi anaemia (FA) repair pathway (Figs 1.6, 1.7), discussed at length in [96] and [148]. The FA pathway is required for direct repair of interstrand DNA crosslinks, which are particularly toxic due to their prevention of the separation of the two strands of ssDNA. The FA genomic instability syndrome is characterised by bone marrow failure, developmental abnormalities and predisposition to cancers, with patient derived cells exhibiting chromosomal instability [7] and a profound sensitivity to DNA interstrand crosslinking agents such as nitrogen mustards and mitomycin C [21, 87]. With analogy to the other repair pathways already discussed, interstrand crosslink repair by the FA pathway requires recognition of an interstrand crosslink with concomitant activation of checkpoint signaling and recruitment and activation of repair factors. The crosslink itself is then

'unhooked' by endonuclease activity exerted by multiple structure specific endonucleases before removal by the nucleotide excision repair (NER) pathway. The damage site may then be bypassed by translesion DNA synthesis, for example by POL ζ or POLN, with ligation restoring dsDNA (Fig 1.6). Alternatively, the removal of an ICL at the site of a stalled replication fork can lead to the generation of a single-ended DSB which can be repaired by the BIR subpathway of HR (Fig 1.7) [148]. The ICL unhooking and removal process is likely to induce distortion of the DNA double helix, the relief of which will require helicase activity. Recent work has established a physical and functional interaction between the FA complementation group helicase, FANCI and BLM [193] which may provide just such a helicase activity at the site of an ICL. The authors find that FANCI and BLM interact upon a DNA substrate with a polyglycol linkage and suggest that this interaction may enable the specific unwinding of the DNA duplex in the lesion bearing region.

The study of patients with FA has allowed the identification of at least fourteen FA genes (termed FANCA) and more are being discovered as our understanding of DNA repair and genomics advances. Some key members of the family are discussed below, although very few have had any enzymatic activity ascribed to them. The initial events following DNA damage sensing are the recruitment of the FA core complex, comprising FANCA,-B,-C,-E,-F,-G,-M, which harbours E3 ubiquitin ligase activity responsible for the mono-ubiquitination of FANCD2 and FANCI. This single modification of

FANCD2 is required for a functional FA pathway and governs its colocalisation with BRCA1 in response to damage induced foci [56], as well as serving to recruit the FA nuclease, FAN1 [123, 105, 183] and translesion polymerase, POLN (see below) [37].

Of central importance within the FA pathway is the highly versatile FANCM. A member of the FA core complex, FANCM facilitates core complex formation on chromatin in response to DNA damage as well as being implicated in FANCD2 monoubiquitination and the checkpoint response to interstrand crosslinks [131, 145, 81]. Notably, FANCM is also one of only two proteins known in the genome that code for both a DNA translocase and a nuclease in the same polypeptide chain (the other being XPF) [6] and can bind synthetic Holliday junctions and replication forks as well as harbouring branch migration activity [57]. Intriguingly, FANCM-deficient cells show a phenotype reminiscent of Bloom syndrome cells with increased sister chromatid exchange [22]. This similarity has been explained by the ability of FANCM to interact with a BLM-containing complex and target it to replication forks [36].

FA research is a fertile area, with a host of new pathway members being discovered recently. A key new FA component is the FAN1 nuclease responsible for the second of two incision steps required to complete the unhooking of a crosslink - the first being performed by MUS81-EME1. The FAN1 nuclease was identified by sequence conservation with prokaryotic ubiquitin binding DNA repair associated proteins, interaction with MLH1, as well as

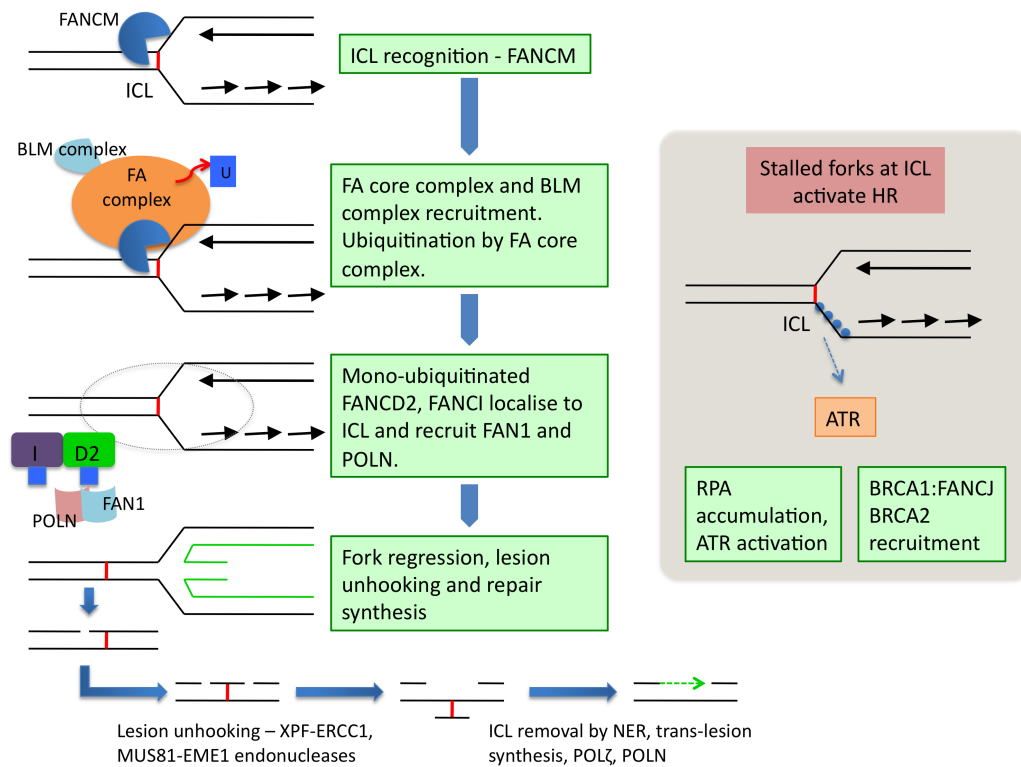


Figure 1.6: Interstrand crosslink repair by the FA pathway and (*inset*) FA coupled HR. An ICL is recognised by FANCM, allowing recruitment of the FA core complex and monoubiquitination of FANCI and FANCD2. The ICL is unhooked by the endonuclease activities of MUS81-EME1 and XPF-ERCC1 and removed by NER and dsDNA restored by translesion synthesis (e.g. by POL ζ) and subsequent ligation. Alternatively (*inset*) HR may be activated following fork stalling by the accumulation of ssDNA-RPA activating ATR.

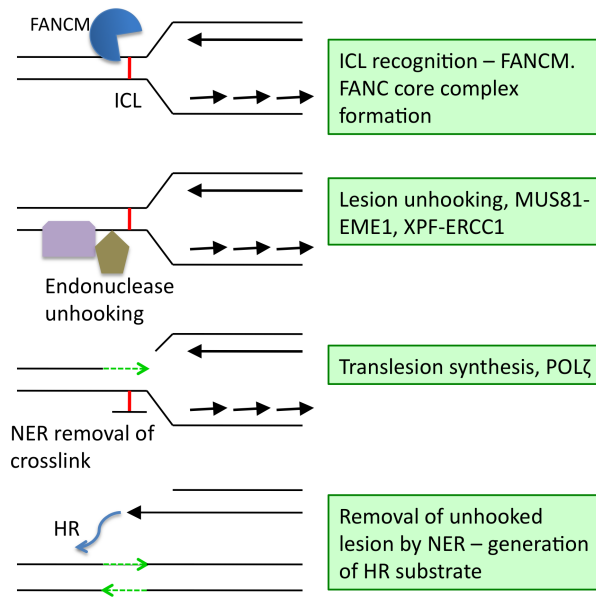


Figure 1.7: Interstrand crosslink repair and HR. ICL repair at a stalled replication fork may proceed initially via the FA repair pathway with lesion recognition and FA core complex assembly as shown in Fig 1.6. The lesion unhooking mediated by the endonuclease activities of MUS81-EME1 and XPF-ERCC1, ICL removal by NER and trans-lesion synthesis (e.g. by POLζ) then leads to the generation of a single ended DSB. This structure may act as a substrate for HR for the completion of repair via the BIR pathway (Fig 1.5).

in a genetic screen for proteins involved in repair of interstrand crosslinks [123, 105, 183]. FAN1 has been shown in these initial studies to be required for interstrand crosslink repair, to be recruited to sites of DNA damage via interaction with monoubiquitinated FANCD2, and to possess 5' flap endonuclease activity and 5' exonuclease activity.

With recombinogenic structures being formed as intermediates of FA pathway repair, for example via replication fork regression after stalling, one could expect some interplay with HR. This expectation is increasingly being realised as the FANCM interaction with the BLM helicase demonstrates. Other notable additions to the FA pathway that emphasise the crosstalk with HR include: the SLX4 nuclease - FANCP [99, 190], BRCA2/FANCD1 [77], its binding partner PALB2/FANCN [168], the BRCA1 associated helicase BACH1/FANCI [114] and RAD51C, tentatively assigned as FANCO [209].

Recent developments in the FA field are serving to develop not just the knowledge of the mechanism of crosslink repair but also to strengthen the ties between the FA and HR repair pathways, which are increasingly difficult to consider in isolation of each other.

1.2 DNA double strand breaks, their repair and cancer

With the exception of surgery, the majority of cancer treatments exploit the toxic effect of DSBs induced either directly by radiotherapy or indirectly

via replicative stress or the replicative processing of DNA lesions generated by alkylating agents, DNA crosslinkers or topoisomerase poisons [72]. Since DSB induced cell kill is the principle mechanism for so many chemotherapeutics, appreciating how the cell repairs this damage gives us the power to understand existing treatments in more depth and to devise new ones.

It becomes obvious, when considering the literature on hereditary cancers, just how important functional DNA damage repair and repair signaling are in restraining cancer development 1.1. Prominent examples of medical conditions resulting from mutation to a member/members of the DNA damage repair family, which include amongst their symptoms a predisposition to cancer that have been cited throughout this introduction include: Ataxia-telangiectasia, Fanconi anaemia, RIDDLE syndrome, Nijmegen break syndrome and familial breast and ovarian cancers. All of these diseases share a defect in some component of the DSB repair pathways.

Of unique interest is how understanding the molecular basis of breast and ovarian cancers, specifically loss of BRCA1 and BRCA2 function, has led to a novel and highly successful treatment strategy in cancer patients, the exploitation of synthetic lethality (explained below). Pioneering studies in two separate laboratories demonstrated the exquisite ability of inhibitors of the poly-ADP-ribose polymerases (PARPs), in particular PARP1, to specifically kill BRCA1 and BRCA2 deficient cells *in vitro* and BRCA2 deficient tumours *in vivo* [19, 48]. These observations led to the fast tracking of PARP inhibitors into the clinic where they have proven effective in the treatment

of breast and ovarian cancers. This interaction between PARP inhibition (which generates recombinogenic lesions) and BRCA defect is described as synthetic lethal. Either PARP inhibition or BRCA inactivation alone are not lethal, but in combination they lead to the accumulation of DSBs that the cell cannot repair by HR and hence drive toxicity.

Exploiting the non-essential role of PARP in the cell, Olaparib (small molecule PARP inhibitor) treatment as a monotherapy has in a recent Phase I clinical trial shown reduced side effects in comparison with conventional chemotherapy and exhibits antitumour activity in cancer associated with BRCA1 or BRCA2 mutation [53]. A phase II clinical trial with a different small molecule PARP inhibitor (Iniparib) in conjunction with chemotherapy has also shown clinical benefit and increased patient survival in metastatic triple-negative breast cancer without significantly increased toxic effects [156].

The success of PARP inhibitors in the clinic has helped to bolster the acceptance that HR and related repair pathways, such as the FA pathway, are key players in mediating patient responses to numerous chemotherapies [71]. For example, the response of ovarian cancers to platinum based (DNA crosslinking) chemotherapy has been correlated with decreased expression of BRCA1 and FANCF [33, 202] or mutations in BRCA1 and BRCA2 [171, 198]. Furthermore, secondary BRCA1 and BRCA2 mutations in women with somatically mutated BRCA1 and BRCA2 may also be used to predict their response to chemotherapy [155]. An additional HR mediated resistance mechanism to etoposide has been uncovered in small cell lung cancer cells due to

their high RAD51 levels [68]. It seems reasonable to suggest, in light of these discoveries, that specific inhibition of HR may be a useful tool for surmounting tumour resistance to standard DNA damaging chemotherapeutics.

The application of HR inhibitors is beginning to be realised in light of our developing understanding of the mechanism of action of a selection of recently developed, novel, chemotherapeutics. Much has been written of the efficacy of the kinase inhibitor Imatinib in the treatment of chronic myelogenous leukemia and gastrointestinal stromal tumours via its selective inhibition of BCR-ABL, cKIT and PDGFR tyrosine kinases, however there is an increasing body of evidence to suggest that Imatinib also impairs HR. Imatinib reduces RAD51 expression levels and CTIP activation (and accordingly resection) in BCR-ABL expressing cells [25, 173].

The impairment of HR by kinase inhibition is not unique to Imatinib, with both Erlotinib and Gefitinib (targeting EGFR tyrosine kinase domains) being shown to reduce HR activity, the latter via reducing RAD51 mRNA levels when used in combination with DNA damaging chemotherapeutics [112, 100]. Nor is this phenomenon restricted to the inhibition of signaling pathways. Drugs with proven anti-tumour activity targeting a diverse group of proteins that influence protein stability and chromatin dynamics have all been shown to influence HR. Both the proteasome inhibitor Bortezomib/Velcade and the heatshock-protein 90 (HSP90) inhibitor 17-AAG influence the stability of recombination proteins, RAD51 and BRCA2 respectively [150, 154]. Both these agents have been successfully used in the clinic with favourable

responses in patients: Bortezomib for multiple myeloma and 17-AAG in combination with Trastuzumab in HER2 positive breast cancers [175, 140].

Chromatin modification also appears to be a relevant clinical target for cancer treatment. The broad spectrum histone deacetylase (HDAC) inhibitor PCI-24781 shows promising antitumour activity in combination with radiation *in vitro* and in human xenograft models and is currently in ongoing clinical trials for its antitumour properties [1, 120, 201]. In parallel to those examples listed above, PCI-24781 treatment reduces RAD51 expression levels, leading to HR inhibition [1, 120]. Supportive of the information so far gathered pertaining to PCI-24781 is the observation that both HDAC9 and HDAC10 are involved in HR [104].

Though not necessarily the primary mechanism for the observed anti-tumour effects of these drugs in the clinic, it is intriguing that they share the ability to limit HR activity in cells, perhaps explaining their utility in combination with DSB inducing chemotherapeutics. This inhibition of repair is also brought about in most cases through similar mechanisms - regulating the expression levels/stability of the core HR factors RAD51 and BRCA2, suggesting a possible, novel, therapeutic target.

1.3 Project aims

In light of the realisation that HR is crucial for organismal development and the day to day maintenance of genomic integrity, and given its pivotal role in cancer development and perhaps even treatment, it is of particular

interest to fully understand all the factors that influence successful HR repair. Furthermore, it may be of use to preclinical and eventually clinical studies to discover and develop compounds that are capable of specifically inhibiting HR. With these aspirations in mind, my project has been targeted towards two specific aims:

1. The identification of novel proteins involved in homologous recombination.
2. The identification of small molecule inhibitors of homologous recombination.

1.3.1 The identification of novel proteins involved in homologous recombination

HR is complex, highly regulated and comprises a host of different proteins acting at multiple stages throughout the cell. It would be naïve to assume that we have a complete knowledge of such an intricate system and as such, new players in HR and related pathways are being described with some regularity. There are different approaches to identify potential HR proteins, notable successes in describing new DNA repair proteins have come from genetic approaches working on heritable conditions predisposing patients to cancer and demonstrating hypersensitivity to DNA damaging agents. An alternative approach is to exploit recent developments in RNA interference (RNAi) knockdown technologies and employ high-throughput screens aimed at dissecting out those proteins that are required for repair. RNAi relies on the intracellular processing of long, double stranded RNAs by the DICER endonuclease to generate short interfering RNAs (siRNAs) of 21-22 nucleotides in length, which, through complementarity to cellular mRNA, mediate specific degradation of their target gene transcripts [52, 14, 44].

I opted to exploit RNAi to screen for novel HR proteins and accordingly

set out to develop a high throughput RNAi compatible cellular assay capable of identifying proteins required for HR. This approach draws on a number of caveats: (i) the availability of a specific assay for HR activity, (ii) its suitability for manipulation by RNAi and (iii) its scalability for high-throughput screening. All these conditions had to be satisfied before running the screen as will be detailed in the subsequent chapters of this thesis.

RNAi screening, though a relatively new science, is not short of successful examples, even within the DNA repair field [183, 85, 182, 121, 133, 161]. Numerous groups have identified novel DNA repair proteins through RNAi screens and though the assays differ markedly, the results speak for themselves. Some illustrative examples applying a variety of RNAi technologies and readout assays are provided below.

Perhaps the most straightforward RNAi assay involves monitoring RNAi mediated changes to cell growth/survival. Reliable, high-throughput cell survival assays, often relying on luminescence or fluorescence, give easily interpretable data. Such assays may be used to identify genes required for resistance or sensitivity to DNA damaging agents; Lord *et al* [121] used such a set up with an siRNA library to identify novel determinants of PARP inhibitor sensitivity. Analogous studies have used cells stably infected with viral particles bearing shRNAs. Cells are infected with pools containing thousands of barcoded short-hairpin RNAs (shRNAs, see below for further explanation) and left to grow in the presence or absence of DNA damaging agents. Within the cell population, shRNAs specifically depleted in response to treatment

may be identified by microarrays for the barcoded shRNAs. Such DNA-damaging agent dependent shRNA depletion identifies those shRNAs that may target the knockdown of candidate DNA repair gene transcripts. Genes responsible for resistance to IR and camptothecin have been characterised in such studies [183, 85]. Other screens have exploited high-throughput microscopy of immuno-fluorescent readouts to identify genes required for cell cycle arrest [31, 133]. These studies used antibodies to phosphorylated histone H3 as a marker for mitotic cells and for the subsequent calculation of mitotic index. In this way they were able to identify novel proteins required for the S-phase (RHINO, as part of the 9-1-1 complex [31]) and G2-M (BRCA2-PALB2 [133] cell cycle checkpoints

The above summary serves to focus attention on two important considerations for RNAi screens. Firstly, the RNAi technology to be used and secondly, the assay readout. Whilst the latter is down to the imagination and resources available to the individual researcher, the selection of an RNAi technology is more restricted. The primary methods of introducing short RNAs into a cell have all been used in the above examples and employ siRNA, shRNA and endoribonuclease prepared siRNA (esiRNA). Most RNAi technologies use a pool of multiple RNAs against each gene target to maximise transcript knockdown whilst minimising any possible off-target effects of any members of the pool.

Perhaps the most straightforward to use, siRNAs are duplex RNA oligonucleotides requiring no cellular processing that may be transfected into a cell

using standard liposome based methodologies. Alternatively, shRNAs may be encoded into a plasmid and expressed following transfection or viral infection of DNA constructs and subsequent transcription. The shRNA undergoes limited processing to remove the hairpin region and reveal an siRNA within the cell. Finally, a large pool of siRNAs can be generated from a long double stranded RNA *in vitro* by endoribonuclease activity. Such a pool of esiRNAs provides many siRNAs capable of targeting a gene of interest and goes some way to mitigating the deleterious off target effects that may be seen by using one or a few siRNAs against a particular gene [224].

Each RNAi technology offers its own specific advantages and disadvantages, which must be considered when interpreting screen data from any RNAi study. Briefly, simple transfection of siRNA is easy to control but gives only a transient protein knockdown, whilst stably integrated, inducible shRNAs may provide more stable knockdown. Certain advantages may be conferred to RNAi experiments by using a viral system to infect cells with shRNAs. Such systems have the ability to generate a large pool of viral particles representing a genome-wide RNAi library to infect a population of cells; see [85, 183] for examples. Barcoding of the shRNAs provides each shRNA with a unique nucleotide sequence which may be specifically identified with a complementary sequence upon a custom micro-array. The barcoding system thus allows highthroughput micro-array based analysis of shRNA screen data. Thousands of shRNA bearing viral particles may be used to infect one population of cells in a single experiment alleviating the problem of thou-

sands of single transfections required for an equivalent siRNA based study. Analysis of the whole cell population and comparison with other populations (such as drug treated) is then possible using the micro-array technology. As described above, both siRNA and shRNA technologies may suffer from off-target effects, as only a limited number of sequences are used to target each gene of interest. esiRNA avoids this problem by having large numbers of siRNAs against each gene. This does not remove the possibility of off-target effects but prevents the dominant effect of a small number of off-target effects in a small siRNA pool from excessively influencing results.

1.3.2 The identification of small molecule inhibitors of homologous recombination

The identification of novel small molecule inhibitors of any pathway is a non-trivial task. Broadly, there are two opposing techniques that may be relied upon; either cell based or biochemical screens. I opted for the former. Furthermore, since the requirement for high-throughput compatibility is shared with the proposed RNAi screen, the same assay may be used for both the RNAi and small molecule screens.

High-throughput screens inevitably involve compromise, by electing to perform a cell-based as opposed to biochemical screen, it is immediately apparent that defining the target of any compounds selected from the screen will be far from straightforward. Bearing this in mind, it is important to note some specific advantages of a cell-based screen, particularly applicable

to the search for HR inhibitors, not offered by a biochemical counterpart.

Firstly, where HR is concerned, there is such a plethora of key players that to limit oneself to a single target in the search for an effective HR inhibitor would be to miss inhibitory opportunities elsewhere in the pathway. The diversity of the targets of those drugs, which have been shown to inhibit HR (detailed above) reinforces this point. Secondly, by definition, any compound that has an effect in a cell based assay already possesses the necessary chemical properties (solubility, partition coefficient) to penetrate the cell membrane and retain stability long enough to exert an effect. Such properties will not necessarily be selected for in a biochemical assay and whilst an investigator may well discover novel, potent and selective compounds for their given target, these will be of no practical use if they are not cell penetrable and metabolically stable. Just such a situation has been played out in the search for selective inhibitors of members of the histone demethylase family by biochemical screening, with numerous promising hit compounds being unable to penetrate into the cell [N.Rose, C.Schofield - personal communication].

In addition to those compounds that indirectly affect HR reviewed above, there are some recent examples of compounds successfully discovered/developed as specific HR inhibitors. These studies have employed a range of techniques that are illustrative of the spectrum of approaches that may be engaged to identify inhibitors of such a complex pathway. The work of Hall *et al* [65] has exploited the knowledge that the BRC domains of BRCA2 bind to RAD51

to guide the design of BRC domain based peptide aptamers that when ectopically expressed in *Trypanosoma brucei*, both inhibit DNA damage repair and sensitise to DNA damaging agents. Other approaches have relied upon biochemical activity assays. The identification of a specific inhibitor of the RAD51 recombinase [80] used an *in vitro* assay with recombinant RAD51 employed to displace, with an unlabelled ssDNA, a quencher labelled ssDNA from its complementary, fluor labelled strand. Successful RAD51 strand displacement was thus detected by the appearance of fluorescence. An *in vitro* WRN helicase activity assay [2] has also been used for the discovery of specific WRN helicase inhibitors capable of sensitising cells to DNA damage inducing agents. A further alternative has applied a *Xenopus laevis* egg extract system recapitulating the ATM and MRN dependent response to DNA damage to successfully identify the MRN inhibitor Mirin [43]. Finally, a cell γ H2AX focus formation assay has yielded inhibitors of the early stages of the DNA damage response [98]. Whilst still not yet clinically applicable, these studies serve as a proof of principle for the successful application of a wide spectrum of techniques to the task of developing specific inhibitors of homologous recombination.

Table 1.1: DNA repair and human disease. Disorders caused by mutations in DNA repair proteins, together with the DNA repair pathways within which they function. With reference to [27].

Disorder	Mutated protein(s)	Pathway(s) effected
Ataxia telangiectasia	ATM	DNA damage signaling
Ataxia telangiectasia-like disorder	MRE11	DNA damage signaling, HR, alt-NHEJ
Bloom syndrome	BLM	HR
Breast cancer, ovarian cancer	BRCA1, BRCA2, RAD51C	HR
Cockayne syndrome	CSA, CSB, XPB, XPD, XPG	TC-NER
Familial breast cancer	ATM, BRCA1, BRCA2, BRIP1, CHK2, NBS1, PALB2, RAD50, RAD51C	HR
Fanconi anemia	FANCA-C, FANCD1, D2, FANCE-G, FANCI, J, L-N	ICL repair, HR
Fanconi anemia like disorder	RAD51C	ICL repair, HR
Hereditary nonpolyposis colorectal cancer	MSH2, MSH6, MLH1, MMR PMS2	

Li-Fraumeni drome	syn-	TP53	DNA damage signaling
Ligase I syndrome		LIG1	NER, SSB repair
Ligase IV syndrome		LIGIV	NHEJ
Nijmegen syndrome	breakage	NBS1	DNA damage signaling
Nijmegen age disorder	break- syndrome-like	RAD50	DNA damage signaling
Radiosensitive combined ficiency	severe immunode-	ARTEMIS	NHEJ
RIDDLE syndrome		RNF168	DNA damage signaling
Rothmund syndrome	Thomson	RECQL4	BER, HR
Seckel syndrome		ATR	DNA damage signaling
Werner syndrome		WRN	BER, HR
Xeroderma sum	pigmento-	XPA-G, POLH	NER

Chapter 2

Materials and Methods

Unless otherwise stated, all compounds sourced commercially were purchased from Sigma. PCI-24781 was acquired by MTA from Pharmacyclics.

2.1 Mammalian cell culture

Cell maintenance

With reference to Table 2.2, all cell lines were grown under identical conditions in plasticware supplied by Corning. All cells were maintained in T175 flasks in 20 ml Dulbeccos modified minimal essential medium (DMEM) (Gibco, Invitrogen) supplemented with 10% foetal bovine serum (FBS - Gibco, Invitrogen), 100 U penicillin, and 0.1 mg/ml streptomycin (referred to hence as complete media). Cells were grown at 37°C in a humidified atmosphere with 5% carbon dioxide and 20% oxygen.

Cells were split every 2-4 days (or when near 100% confluency) by as-

Table 2.1: Commonly used buffers throughout this study.

buffer	
lysis buffer	2X lysis buffer (5ml), 10mM NaF (100 μ l), 5mM NaPP _i (500 μ l), H ₂ O (3ml), 1.4ml 7X protease inhibitor (Roche)
2X lysis buffer	25mM Tris-HCl (pH7.6), 150mM NaCl, 1% NP-40, 1% sodium-deoxycholate, 0.1% SDS
10X MOPS	0.2 M MOPS, 10 mM EDTA, 50 mM NaOAc
1X PBS	1 X phosphate buffered saline tablet per 100ml, 0.01M
Ponceau Red	0.2% Ponceau red and 1% acetic acid in H ₂ O
1X TBS	25 mM Tris, 150 mM NaCl, 2 mM KCl in H ₂ O, pH8.0
TBS-T	1X TBS, 0.1% (v/v) Tween
transfer buffer	1X tris-glycine buffer, 10% methanol (v/v)
1X tris-glycine	25 mM Tris, 192 mM glycine in H ₂ O, pH 8.3
4% PFA	4% paraformaldehyde (w/v) in 1X PBS, dissolved overnight at 70°C
4 % PFA, 0.1% Triton-X	4% PFA in 1X PBS with 0.1% Triton-X (v/v)
0.3% Triton-X	0.3% Triton-X (v/v) in 1X PBS
3% BSA	3% bovine serum albumin in 1X PBS

piration of the existing media followed by washing with 10ml 1X PBS and once with 2ml 1X trypsin-EDTA (0.25% trypsin, 0.53mM EDTA) solution (made up in 1X PBS from 10X stock; SAFC). Following 5 minutes incubation at 37°C, trypsinised cells were re-suspended in complete media ready for seeding.

Thawing and freezing

Liquid nitrogen stocks were rapidly thawed at 37°C for 2 minutes, and the cell suspension diluted with 10 ml complete media. The cells were pelleted by centrifugation at 1000g for 5 minutes, the supernatant aspirated and the cell pellet resuspended with 10ml complete media at 37°C and transferred to a T25 flask.

Cell lines to be frozen were grown to around 90% confluency in T175 flasks and trypsinised as described above. The cells were re-suspended in 15 ml complete DMEM containing 10% (v/v) tissue culture ready DMSO. 1.5 ml aliquots were then prepared in cryotubes and frozen at -80°C overnight before being transferred into liquid nitrogen.

Preparation of cell line clones

Two hundred cells were seeded in a 10cm dish and left to grow for 12-14 days in selective, complete DMEM until visible colonies of cells had formed. Individual, discreet, colonies were then selected, separately trypsinised and transferred to 24-well plate where they were grown in selective DMEM until confluent. Confluent cells were then trypsinised and transferred to T25 flasks

Table 2.2: Cell lines used throughout this study, together with the species, tissue of origin and source from which they were derived.

cell line	species	tissue	source
U2OS	human	osteosarcoma	ATCC
U2OS-DRGFP cl.1 (UGFP1)		osteosarcoma	U2OS
MRC5-DRGFP (MGFP)		lung fibroblast	H.Bryant
MRC5-DRGFP cl.6 (MGFP6)		lung fibroblast	MRC5-DRGFP
A11 (PARP1 ^{-/-})	mouse	embryonic fibroblast	N.Curtin
A19 (PARP1 ^{wt})		embryonic fibroblast	N.Curtin

for expansion and further work.

Cell cycle synchronisation

For preparation of S-phase cells, cells were first synchronised in G1 phase by overnight treatment with 350 μ M mimosine (dissolved in complete DMEM). Cells were then released from G1 block by washing with 1X PBS and addition of complete DMEM. Cells were isolated at 6 hours following mimosine release to allow for entry into S-phase.

Cell cycle analysis

Highthroughput cell cycle analysis in 96-well plate was conducted by cell sorting of propidium iodide (PI) stained cells using the Becton Dickinson

FACSCalibur cytometer. All volumes are per well. Prior to cytometry, cells were trypsinised *in situ* with 20 μ l 3X trypsin (0.75% trypsin, 1.59mM EDTA) for 20mins at 37° followed by permeabilisation and PI staining by addition of 40 μ l serum and antibiotic free medium containing 0.75% (v/v) Tween and 0.15 μ l of 1mg/ml PI.

Low throughput cell cycle analysis was conducted on BrdU labelled and PI stained cells by flow cytometry. Cells grown in 6-well plates were first labelled with 20 μ M BrdU for 20 minutes followed by trypsinisation using the standard cell culture trypsinisation protocol. Trypsinised cells were collected and harvested by centrifugation at 1000g for 5 minutes before resuspension in 100 μ l 1X PBS and fixation by dropwise addition, with mixing, of ice cold 70% ethanol.

Fixed cells were then processed for FACS by Dr M. Woodcock, as follows: centrifugation at 700g for 5 minutes followed by resuspension in 1ml of 2M HCl containing 0.1mg/ml pepsin and incubation at room temperature for 20 minutes. Centrifugation was repeated at 700g for 5 minutes prior to washing with 1X PBS before a further centrifugation step and resuspension of cells in 1ml 1X PBS containing 0.5% FBS and 0.5% Tween and 20 μ l of 10mg/ml RNase. After an additional centrifugation, cells were resuspended in 100 μ l 1X PBS containing 2% FBS and 1 μ l mouse anti-BrdU monoclonal antibody (Becton Dickinson) and incubated for 90 minutes at room temperature. Secondary antibody staining was conducted by addition of 2 μ l goat anti-mouse Alexa 488 antibody (Invitrogen) and incubation for 60 minutes at room tem-

perature in the dark. Antibody-stained cells were centrifuged at 700g for 5 minutes before resuspension in 2ml 1X PBS containing 5 μ l 1mg/ml PI, incubated for 30 minutes at room temperature and then analysed by flow cytometry.

2.2 Transfections

PEI plasmid transfections

For the screen optimisation, running and initial post-screen work with the DR-GFP assay, the transfection reagent polyethylenimine (PEI) was used. A 100mg/ml stock was made up in water and diluted to a 1mg/ml working stock, each stock was filter sterilised and the pH adjusted to 7.0 with 5M HCl. All transfection mixtures were made in a tissue culture hood.

With reference to Table 2.5, cells at 80-90% confluency in 96-well plate were transfected as follows: Per well, PEI was added to 50 μ l of optiMEM (Gibco, Invitrogen), vortexed and left for 5 minutes at room temperature. To this mixture was added plasmid DNA followed by vortexing. The PEI-plasmid-optiMEM mixture was left to incubate for 25 minutes at room temperature allowing PEI:DNA complexes to form, before the 50 μ l solution was added, dropwise to cells in 100 μ l antibiotic free DMEM. 24 hours following transfection, the cells were washed with 1X PBS before addition of 200 μ l complete DMEM. Depending on the assay, cells were analysed 48 hours following transfection. In those cases where 384-well plates were used, the

Table 2.3: Lipofectamine 2000 transfection conditions used in this study with Lipofectamine abbreviated to 'lipo' and optiMEM to 'opti'.

plate	cell no. (k)	lipo(μ l)	opti(μ l)	DNA(μ g)	opti(μ l)	DMEM (μ l)
6 well	350	10	250	4	250	2000
24 well	75-100	2	50	0.8	50	500
96 well	8-15	0.5	25	0.2	25	100

protocol above was adapted by changing the reagent volumes and amounts (per well) as follows; cells were resuspended in 30 μ l antibiotic free DMEM and added to optiMEM 15 μ l containing DNA (0.09 μ g) and PEI (0.1125 μ g) made up in the same manner as the 96-well plate protocol.

Lipofectamine 2000 plasmid transfections

The majority of post-screening work was conducted with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. This was deemed inappropriate for the screen due to its expense. Transfections were conducted in 96-, 24- and 6- well plates, depending on the experiment. The reagent volumes varied depending on the plate/dish used and are shown in Table 2.3. Cells were seeded 24 hours before transfection, or reverse transfected with siRNA 48 hours before transfection (see below). The transfection mix was produced in two stages, with separate mixing of the Lipofectamine 2000-optiMEM and DNA-optiMEM components before incubation for 5 minutes

Table 2.4: siRNA reverse transfection conditions used in this study with DharmaFECT abbreviated to 'DF' and optiMEM to 'opti'.

plate	cell no. (k)	cell vol. (μ l)	DF mix (μ l)	siRNA(μ l)	opti(μ l)
10cm	1300	4000	500	25	475
6 well	350	1600	200	10	190
96 well	8-12	80	10	0.5	9.5

at room temperature. Each of these two mixtures was then combined, vortexed and left to incubate for a further 25 minutes at room temperature before being added dropwise to cells, which had been placed in antibiotic free DMEM immediately prior to transfection.

siRNA reverse transfections

For all siRNA transfections, the reverse transfection protocol was used; siRNA-transfection reagent mixture is first plated out with subsequent addition of trypsinised, suspended cells. This serves to reduce the total time of a 'forward' transfection protocol by 24 hours as cells do not need to be plated in advance. In all cases DharmaFECT (Dharmacon) transfection reagents were used, specifically DharmaFECT 1 or 2. In either case, an identical protocol was used.

Similarly to the Lipofectamine 2000 plasmid transfections, transfections were conducted in 96- and 6- well plates as well as 10cm dishes, depending on

the experiment (Table 2.4). The transfection mix was produced in two stages, with separate mixing of the DharmaFECT-optiMEM and siRNA-optiMEM components before incubation for 5 minutes at room temperature. Each of these two mixtures was then combined, vortexed and left to incubate for a further 25 minutes at room temperature before being plated into empty wells. Trypsinised cells were then resuspended and added in the relevant volume of antibiotic free DMEM to the transfection mix.

2.3 DR-GFP assay

The DR-GFP assay (Fig 2.1 (a)) was run in accordance with the original publication [165], but modified for 96-well plate. The DR-GFP construct contains two GFP coding regions; one, full length sequence bearing a premature stop codon and an I-SceI endonuclease target sequence and the second, a truncated sequence. Induction of a site-specific double strand break by the I-SceI endonuclease in the first GFP sequence provides a DSB for HR. The HR machinery may then use the second, truncated, GFP sequence as a repair template to generate full length GFP and produce fluorescent cells.

With reference to Fig 2.1 (b), cells bearing the DR-GFP reporter (e.g. MGFP6, UGFP1 cells) were first treated with compounds or siRNAs and incubated before a site specific double strand break was introduced into the reporter. This was achieved by transfection of the cells with a vector encoding the rare cutting I-SceI endonuclease. 24 hours later, cells were washed with 100 μ l 1X PBS and 200 μ l complete DMEM was added. Two days fol-

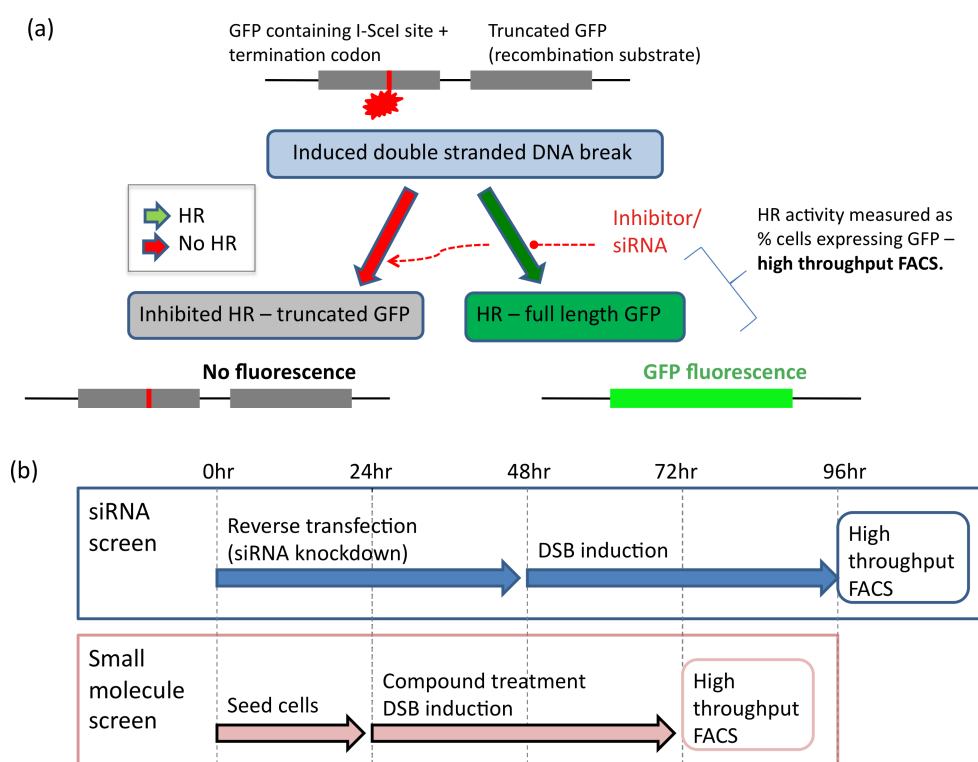


Figure 2.1: **(a)** The DR-GFP HR assay. The GFP reporter consists of two GFP sequences, the first bearing a premature stop codon within an I-SceI endonuclease cutting site and the second a truncated GFP sequence. Introduction of a DSB within the first DNA sequence is achieved by transfection with the I-SceI endonuclease. Successful HR recombines the full length GFP gene and fluorescence results. The siRNA and small molecule treatment protocols are shown in **(b)**. For the siRNA protocol, protein knockdown was achieved by reverse siRNA transfection (48 hours) followed by I-SceI transfection. GFP-positive cells were counted by FACS 48 hours after I-SceI treatment. For the small molecule protocol, cells were seeded 24 hours before a combined compound treatment and I-SceI transfection and GFP-positive cells were counted by FACS 48 hours thence.

Table 2.5: DR-GFP assay conditions used in this study with respect to siRNA and I-SceI transfections. The DharmaFECT transfection reagent is abbreviated to 'DF'.

cell line	[siRNA] (nM)	% DF (v/v)	DF1/2	I-SceI (μ g)	PEI (μ g)
MGFP	50nM, 20nM	0.100	DF1	0.25	0.50
MGFP6	10nM	0.075	DF2	0.25	0.25
UGFP1	10nM	0.100	DF1	0.25	0.25

Following I-SceI transfection, the cells were trypsinised as follows: following washing with 100 μ l 1X PBS, 30 μ l 3X trypsin was left on the cells for 20 minutes at 37°C, cells were resuspended either in 1X PBS or 4% PFA. Cells were then analysed by flow cytometry in a Becton Dickinson FACScalibur FACS machine with high-throughput capability. CellQuest Pro software was used to select healthy cells (based on their side- and forward- scatter properties) for gating and quantifying the percentage of GFP-expressing cells. The percentage of green fluorescent cells acts as a readout of HR efficiency. Any compound or siRNA that inhibits HR will lead to a reduction in the percentage of GFP-expressing cells as measured by FACS. For 384-well plate experiments, trypsinisation was as above but with the following changes in volumes: 3X trypsin at 5 μ l and 1X PBS or 4% PFA at 5 μ l. Transfection conditions for the DR-GFP assay together with siRNA transfection conditions for each cell line are given in Table 2.5.

2.4 Clonogenic and cell viability assays

Clonogenic survival assays

All clonogenic survival assays were conducted with siRNA reverse transfected cells according to the 6-well plate protocol outlined in Table 2.4. 24 hours following siRNA transfection, cells were trypsinised and seeded in 6-well tissue culture plates in 2.5ml of complete DMEM. Depending on the concentration of drug/dose of ionising radiation being applied, non-targeting siRNA transfected cells were seeded at 200, 400 or 600 cells/well. Test siRNA transfected cells were seeded at 400 cells/well and RAD51 siRNA transfected cells were seeded at 2500 cells per well due to intrinsically poor colony formation efficiency. siRNA transfected, plated cells were left for 24 hours before drug or ionising radiation treatment. Drug treated cells were washed after 24 hours with 1X PBS and 2.5ml fresh, complete DMEM was applied. Cells were then left to grow for 10 days before removal from the incubator, washing with 1X PBS and staining with Brilliant Blue (Sigma) for around 10 minutes at room temperature (0.5ml of stain per well of a 6 well plate). Following staining, excess stain was removed and plates were washed in excess tap water ready for scoring of those colonies with more than 50 cells by hand.

Gamma-ray exposures were performed using a GSR D1 137Cs -ray irradiator (Gamma-Service Medical GmbH, Leipzig, Germany) at a dose rate of 1.7 Gy/min.

Cell viability assay

Cell viability in 96-well plate was estimated following a 1 hour incubation at 37°C of adherent cells with 10 $\mu\text{g}/\text{ml}$ resazurin sodium salt dissolved from a 100mg/ml stock in fresh complete DMEM. The resazurin dye is reduced by NADH and NADPH produced by viable cells to yield a fluorescent product, which is measured by spectrophotometry. Fluorescence intensity correlates with viable cell number. Fluorometric analysis was performed at Ex530nm Em590nm using a Perkin Elmer Envision plate reader. In all cases, blank controls were subtracted and viability expressed as a percentage of the untreated control.

Drug treatments for the cell viability assays were performed 24 hours following cell seeding, with viability timepoints varying from 24- to 144-hours post compound treatment dependent on the assay. DNA damaging drugs were left on the cells until the reading of the first timepoint (i.e. plate washing and addition of resazurin).

2.5 Gels and blotting

2.5.1 Western blotting

Whole cell extract preparation

Whole cell extracts were prepared from 96-well plated cells using 4 wells per condition. Wells were washed with 100 μl 1X PBS before addition of 20 μl premade lysis buffer, to which protease inhibitors had been added (Complete

mini - Roche). Lysis buffer was made up and aliquoted into 429 μ l aliquots and frozen. Aliquots were thawed and 71 μ l protease inhibitor (7X, made up from Complete Mini protease inhibitor tablets, Roche) was added before use.

Lysed cells were pooled from their respective wells and sonicated for 20 minutes on ice, before centrifugation at 13000rpm and 4°C for 15 minutes to remove cell debris. 19.5 μ l of the supernatant was then mixed with 7.5 μ l nuPAGE LDS loading buffer (Invitrogen) and 3 μ l 0.5M dithiothreitol before protein denaturation by incubation at 90°C for 5 minutes.

SDS-PAGE and Western transfer

Prepared samples (20 μ g as measured by ND-1000 spectrophotometer [Nanodrop])) were loaded onto pre-cast 4-12% gradient Bis-Tris gels (Invitrogen) alongside 5 μ l of SeaBlue Plus2 protein marker ladder (Invitrogen). Gels were loaded into tanks (Invitrogen) containing 1x NuPAGE MOPS SDS running buffer (Invitrogen) to which 500 μ l antioxidant (NuPAGE Antioxidant (NP0005), Invitrogen) had been added, in the inner chamber. The gel was run at 170V for 70 minutes.

Following electrophoresis, the transfer was setup by overlaying a 1X transfer buffer soaked sponge, with 2 similarly soaked pieces of Whatman paper, which were flattened with a roller to ensure even protein transfer. The gel was placed onto the Whatman paper, and covered with a pre-soaked nitrocellulose membrane. The membrane was then covered with two pieces of soaked Whatman paper and a soaked sponge after each layer had been flat-

tened. The sandwiched membrane was loaded into a transfer tank containing 1X transfer buffer and an ice block. Transfer proceeded at 100V for 1 hour. Following transfer, the nitrocellulose membrane was stained to check for even loading by staining with 1ml of Ponceau Red for 1 minute at room temperature. The membrane was then rinsed 5 times for 5 minutes in 1X TBS-T, and blocked by 1 hour incubation at room temperature in 1X TBS-T containing 5% skimmed milk (w/v, Fluka).

Immunodetection

The blocked nitrocellulose membrane was washed 5 minutes in 1X TBS-T (five times) then incubated overnight at 4°C in a solution containing 5% milk and an appropriate dilution of primary antibody (Table 2.6). After 5X 5 minute washes with 1X TBS-T, the membrane was incubated for 1 hour at room temperature in 1X TBS-T containing 5 % milk (w/v) a 10,000 fold dilution of secondary antibody (see Table 2.6).

Table 2.6: Antibodies used for Western blotting.

antigen	antibody ID	animal	clonality	dilution	supplier
c9orf102/RAD26L	S-13	rabbit	polyclonal	1:1000	Santa Cruz
His tag	4D11	mouse	monoclonal	1:1000	Abcam
JMJD1B	6A1-1F5	mouse	monoclonal	1:1000	Cell Signaling
MLH1	G168-15	mouse	monoclonal	1:100	BD Pharmingen
RAD51	H-92	rabbit	polyclonal	1:250, 1:1000	Santa Cruz
SUV3	-	rabbit	polyclonal	1:2000	P.Stepien
α -tubulin	T9026	mouse	monoclonal	1:5000	Sigma
WRN	H-300	rabbit	polyclonal	1:1000	Santa Cruz
mouse IgG	ab6728	rabbit	polyclonal	1:10000	Abcam
rabbit IgG	ab6721	goat	polyclonal	1:10000	Abcam
goat IgG	ab6741	rabbit	polyclonal	1:10000	Abcam

Chemiluminescence detection

Following 5X 5 minutes washing of the membrane with 1X TBS-T, the BM Chemiluminescence kit (Roche) was used according to the manufacturers instructions. Briefly, 1:100 starting reagent B (stabilised H₂O₂) was added to substrate reagent A (buffered solution containing luminol and 4-iodophenol) and mixed, immediately before use. The nitrocellulose membrane was coated with the mixed solution (approximately 1ml/membrane) and incubated at room temperature for 1 minute, before removal of the excess liquid. Incubation of the reaction substrates with the Horseradish peroxidase conjugated secondary antibody leads to production of light, which may be detected with X-ray film. In a darkroom, the membrane was transferred into a Hypercasette (Amersham Biosciences) and overlaid with X-ray film (Fujifilm). The X-ray film was exposed for between 2 seconds to 5 minutes depending on the signal and subsequently developed using a film processor (Konica Minolta SRX-101A).

2.5.2 Northern blotting

Cells were prepared and RNA isolated by the author. RNA electrophoresis, transfer and detection were conducted by Dr Csanad Bachrati, Helleday lab according to a standard protocol (Perkin Elmer, see below).

RNA preparation and electrophoresis

Following sample preparation in 10cm tissue culture dishes, RNA was isolated from the cells using the RNeasy Plus Mini kit (Qiagen), according to the manufacturer's protocols. Briefly, cells were lysed with 600 μ l RLT plus buffer, mixed and homogenised with a syringe and needle and applied to a gDNA Eliminator spin column for centrifugation at 10000g for 30 seconds. The flow-through was retained and 600 μ l 70% ethanol was added, with mixing. This mix was applied to an RNeasy spin column and centrifuged at 10000g for 30 seconds. The flow-through was discarded and 500 μ l RPE buffer was added to the column before centrifugation as above and discarding of the flow-through. This RPE wash step was repeated before elution with 50 μ l RNase-free water (Qiagen). RNA concentration was measured with a nanodrop ND-1000 spectrometer (Nanodrop). 10 μ g of RNA was mixed with 20 μ l loading buffer (see Table 2.7) and RNA was run on a 1% agarose gel containing 0.66M formaldehyde in 1X MOPS buffer (Sigma) together with a ssRNA marker (NEB) in a fume hood.

Northern transfer

Transfer was conducted using a Genescreen membrane according to the manufacturer's protocol and buffers therein (Perkin Elmer, GeneScreen). The RNA gel was soaked in 5 volumes of RNase free water for around 5 minutes (X4). A piece of GeneScreen membrane (Perkin Elmer) was cut to the size of the gel and, once hydrated with water, was soaked in 10X SSC buffer for 15

Table 2.7: RNA gel loading buffer.

loading buffer	
formamide (1.14M)	720 μ l
10X MOPS	160 μ l
formaldehyde (12.3M)	260 μ l
dH ₂ O	180 μ l
80% glycerol	100 μ l
bromophenol blue (0.2% bro-	80 μ l
mophenol blue, 10mM EDTA,	
50 % glycerol in DEPC-treated	
dH ₂ O)	

minutes. Next, a capillary blot was setup with 10X SSC as a transfer solution and transfer continued for around 20 hours. After transfer, the membrane was removed from the gel, rinsed in 2X SSPE buffer and placed (RNA side up) on filter paper for drying. RNA was then fixed to the membrane using a 254nm UV lamp for 1min40s at the surface of the wetted membrane. The formaldehyde reaction was then reversed before probing and detection by baking the membrane for two hours at 80°C.

Probe labeling

The DNA probe (full length c9orf102 EST from Source Bioscience) was labeled using the Rediprime II labeling kit (Amersham). Denatured template was mixed with 5 μ l [α^{32} P] dCTP (50 μ Ci), mixed, spun briefly on a bench top mini-centrifuge before incubation for 10 min at 37°C. Detection was performed using a BioRad phosphoimager.

2.6 Immunofluorescence

All immunofluorescence experiments were carried out in 96-well plates using U2OS cells (the parent cell line of the UGFP1 cells used for the DR-GFP assay described herein), all volumes quoted are per 96-well. Following seeding or reverse transfection of cells and any drug treatment required and with reference to Table 2.8, DNA damage was induced in cells by 24 hour treatment with 50nM camptothecin (CPT), with cells fixed at this 24 hour timepoint in 50 μ l 4% PFA, 0.1% triton-X for 10 minutes following a wash with 100 μ l 1X

Table 2.8: Immunofluorescence treatment protocol for both siRNA and small molecule treatments.

assay	day1	day2	day3	day4
siRNA	reverse transfect 8,000 cells	add media	CPT	fix
small molecule	seed 10,000 cells	compound treat	CPT	fix

PBS. Cells were then permeabilised with 100 μ l 0.3% Triton-X for 10 minutes and washed thrice with 100 μ l 1X PBS before blocking with 50 μ l 3% BSA for 40 minutes. Primary antibodies (against 53BP1, RAD51 and RPA p34 - see Table 2.9) were made up in 3% BSA at 1:1000 and cells were incubated with 50 μ l antibody solution overnight at 4°C. Cells were now washed thrice with 100 μ l 1X PBS before a one hour incubation at room temperature with secondary antibody conjugated to Alexa-488 (Invitrogen) and an additional three washes with 1X PBS wash as above. Cells were then stained with DAPI (1:1000 v/v in 1X PBS), left in 100 μ l 1X PBS and stored at 4°C until imaging.

Automated image capture and analysis was performed using the InCell Analyser 1000 (GE healthcare). For each well of a 96-well plate, 4 images were taken. DAPI positive cell nuclei were detected and only nuclear foci scored. Irregularly shaped (e.g. apoptotic or checkpoint deficient) cells were excluded from the analysis. Cells with greater than 5- (53BP1) or 9- (RPA-p34, RAD51) nuclear foci were scored as positive. These thresholds were

Table 2.9: Antibodies used for immunofluorescence, both primary and secondary, together with their respective dilutions.

antigen	antibody ID	animal	clonality	dilution	supplier
53BP1	A300-272A	rabbit	polyclonal	1:1000	Bethyl
RAD51	H-92	rabbit	polyclonal	1:1000	Santa-Cruz
RPA p-34	9H8	mouse	polyclonal	1:1000	Thermo
alexa-488 α -mouse	A-11001	goat	polyclonal	1:10000	Invitrogen
alexa-488 α -rabbit	A-11034	goat	polyclonal	1:10000	Invitrogen
alexa-568 α -mouse	A-11004	goat	polyclonal	1:10000	Invitrogen

chosen to distinguish cells with induced damage from the background level of foci seen in cells with no induced damage (in this case, no CPT treatment). 53BP1 foci, tended to be larger and fewer in number than both RPA-p34 and RAD51 foci. Alexa 488 secondary antibody imaging was performed at 480nm excitation and 535nm emission, whilst Alexa 568 secondary detection was performed at 565nm excitation and 620nm emission.

2.7 Quantitative-, real time- PCR (qPCR)

mRNA isolation and cDNA preparation

qPCR, from cell lysis to the real time PCR reaction, was carried out using the Power SYBR Green Cells to CT kit (Ambion). Following cell preparation in

96-well plate, cells were lysed by first washing with 100 μ l 1X PBS and then addition of 50 μ l lysis solution to which 1:100 DNase I had been added. The DNase I was inactivated by addition of 5 μ l stop solution and the lysate was subsequently stored at -20°C.

For cDNA preparation, a reverse transcriptase (RT) master mix (50 μ l total), made up with nuclease-free water (Qiagen)) was made containing 25 μ l 2X SYBR RT buffer and 2.5 μ l 20X RT enzyme mix with 5 μ l of the whole cell lysate (preparation outlined above). This mixture was aliquoted into a 100 μ l PCR reaction tube (Axygen) and placed in a Techne TC-512 thermocycler. The reverse transcriptase reaction was carried out under the following conditions; 37°C for 60 minutes, then stopped by incubating at 95°C for 5 minutes. The reaction was then put on hold at 4°C. cDNA was stored at -20°C.

Real time-PCR

A 20 μ l qPCR mix was prepared (in triplicate for each sample) in a 96-well qPCR plate and sealed with a film (Applied Biosystems). Each sample contained; 10 μ l Power SYBR green PCR master mix, mixed forward and reverse primers (see Appendix) at a final concentration of 250nM, 2 μ l cDNA and nuclease-free water. The PCR plate was then loaded into an Applied Biosystems 7500 Fast Real Time PCR machine and run on the following cycle (cycle number in paranthesis); 95°C for 10 minutes (X1), 95°C for 15 seconds followed by 60°C for 1 minute (X40). Δ CT data were exported from

the qPCR instrument and processed using MS Excel where the results were normalised to both the internal GAPDH controls and to NT (non-targeting) siRNA treated samples.

2.8 High-throughput screening

Robotic handling station

All programming of the robotics protocol was conducted by Mr Daniel Ebner, Helleday lab. The PerkinElmer Janus Automated Workstation was used for both siRNA and small molecule treatments. The workstation is enclosed in a tissue culture cabinet, and comprises three principle components; the Flexdrop - a 96-well automated cell seeding head with connected plate stackers, the Verispan - eight independent liquid dispensing tips with connected plate stackers, and the MDT, which is a 96 pin liquid dispensing arm. For the screens undertaken in this study, the Flexdrop and Verispan modules of the robotic handling station were used. The Flexdrop 96-well seeding head was used for steps in the protocol which required the transfer of the same reagent to all wells in a plate, for example the seeding of cells. The Verispan was used when small volumes ($10\mu\text{l}$ and under) needed to be transferred.

2.8.1 siRNA screen

The screen was performed using an siRNA library targeting 579 DNA repair and chromatin remodelling genes. Each gene's transcript was targeted

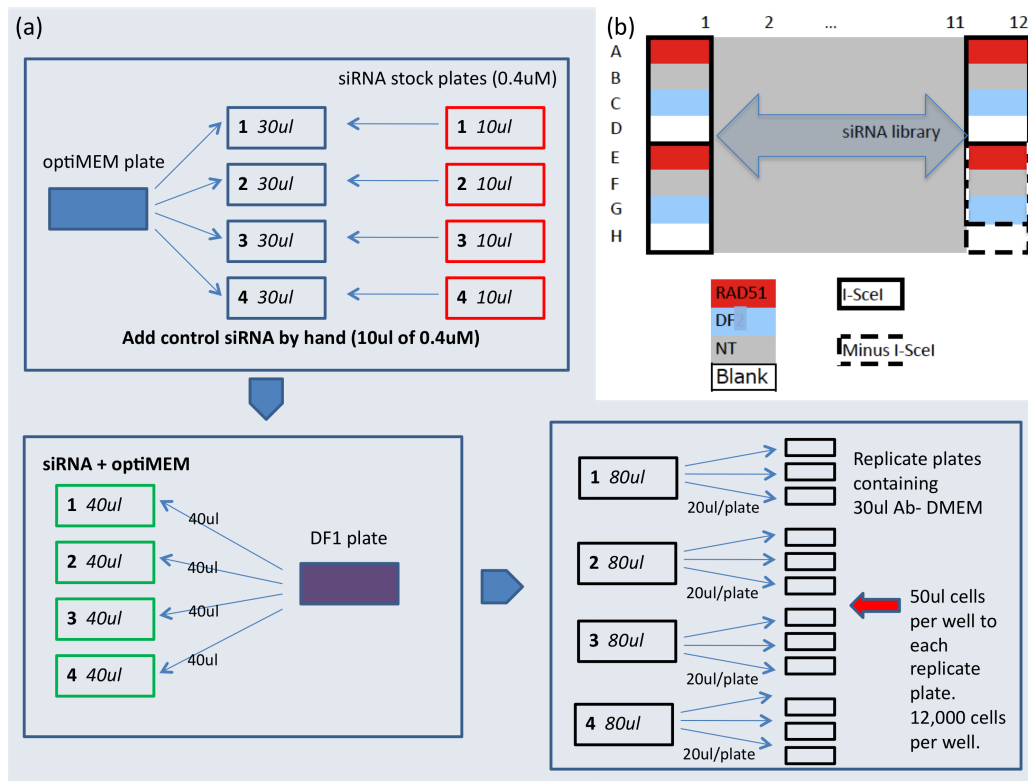


Figure 2.2: siRNA screening by robot. **(a)** The protocol followed by the robotic handler for siRNA transfection. Briefly, optiMEM was plated into four 96-well plates to which siRNA from stock plates was added. These siRNA-optiMEM plates then had optiMEM-DF1 added to them to create 4 transfection reagent plates in which the siRNA-DF1 transfection complexes could form. These transfection reagent plates were then aliquoted into triplicate 96-well plates ready for addition of trypsinised, suspended cells. **(b)** outline of the plate layout, with controls in columns 1 and 12 and the siRNA library in columns 2-11. The layout of the siRNA controls (RAD51, DF only, NT and an optiMEM only blank) is also shown. DF1 = DharmaFECT1 and NT = non-targeting siRNA.

by four separate, distinct siRNAs which were combined into a pool. The library was supplied plated into 96 well plates. Each well contained a pool of four siRNAs each targeting the same gene transcript. The siRNA screen applied the reverse transfection siRNA protocol. In this protocol, trypsinised, suspended cells are pipetted onto the siRNA-DharmaFECT1 transfection reagent which has been pre-dispensed into wells of a 96-well plate. Following optimisation of the siRNA and I-SceI transfection conditions (see Table 2.5), the siRNA screen was setup and run using UGFP1 cells as follows and with reference to Fig 2.1(b): siRNA-DharmaFECT1 complexes were made from the Qiagen DNA damage repair and chromatin modification siRNA library using the robotic handling station, with the siRNA controls added by hand and the reverse transfection protocol was carried out according to Fig 2.2 (a). The following day, the antibiotic-free DMEM that the cells had been seeded in was topped up with 150 μ l complete DMEM by hand. On the third day, I-SceI transfection was carried out by hand, with plates washed with 1X PBS and refreshed with 200 μ l complete medium on the fourth day. Cells were trypsinised for FACS on day five of the assay, for all screens trypsinisation was performed by addition of 20 μ l 3X trypsin and incubation for 20 minutes at 37°C followed by addition of 40 μ l 4% PFA. The screen was run at 10nM and 6nM of siRNA.

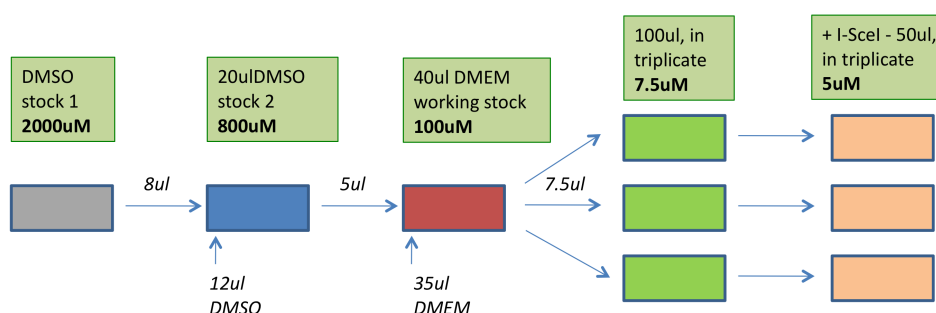


Figure 2.3: Compound (by robot) and I-SceI (by hand) treatment protocol for small molecule screen. Briefly, a DMSO stock plate was made up from the primary compound stocks, from this $800\mu\text{M}$ compound plate, the robot made up a working stock in antibiotic-free DMEM. This working stock was aliquoted, in triplicate to plated cells whilst controls were added by hand - this served to simplify the robotic protocol and increase its speed. The I-SceI transfection mix was then added by hand.

2.8.2 Small molecule screen

With reference to Figures 2.1 (b) and 2.3. Following seeding of 12000 UGFP1 cells in 96-well plates by hand and incubation overnight, the robotic handler was used to wash the cells with 100 μ l 1X PBS before addition of 92.5 μ l antibiotic-free DMEM. To this medium was added 7.5 μ l of a working stock of compound (prepared in antibiotic-free DMEM by the robotic handler). At this point, controls (blank, 0.5% DMSO, 0.4- and 0.6 μ M PCI-24781) were added by hand. Finally, 50 μ l I-SceI transfection mix (0.25 μ g I-SceI, 0.25 μ g PEI in optiMEM) was added by hand. After a 24 hour incubation, the plates were washed by hand with 50 μ l 1X PBS and refreshed with 200 μ l complete DMEM. On the final day of the assay, cells were harvested for FACS as detailed above. Multiple screen runs were required to process all compounds. The kinase inhibitor screen was conducted as outlined above, but due to limited compound number, the drug treatments could be performed by hand.

DR-GFP screen data analysis

For both the siRNA and small molecule screens, the same data analysis protocol was used. Both assays were performed in triplicate, with three separate plates making up the replicates. With reference to Fig 2.4, following raw data (% GFP positive cells) collection, all % GFP cell data was normalised to the mean non-targeting siRNA or DMSO treated wells (for siRNA and small molecule screens, respectively) on a plate by plate basis. This yielded three normalised values for each siRNA or small molecule. The mean of these

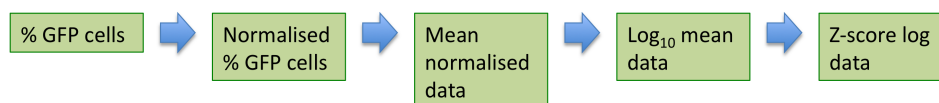


Figure 2.4: Screen data analysis flowchart, from raw data to Z-scores.

values was taken and from this a logarithm (base 10) was calculated. This Log_{10} value was then used for calculating the Z-score (see below).

Z-score

The Z-score is a metric commonly used in high-throughput screening to calculate the statistical significance of each value in a screen. The Z-score is calculated by subtracting the mean of the dataset from every individual value and dividing this number by the standard deviation of the dataset.

The calculated Z-score allows straightforward analysis of a dataset and stratification of hits. Briefly, a Z-score with a value of 1 represents a datapoint that lies one standard deviation away from the mean of the population, a Z-score with a value of 2 is two standard deviations from the mean and so on. Positive Z-scores represent datapoints above the mean and negative, those below the mean. High-throughput biological screening groups commonly use a threshold of a Z-score of two or above (or -2 and below) to define a hit.

In my siRNA screen (DNA damage repair and chromatin remodelling library, Qiagen), due to the relatively small number of siRNAs used (579), a Z-score upper threshold of -1.5 was selected, with any siRNA below this value defined as a preliminary hit i.e. leading to a decrease in HR activity. Although not statistically rigorous, this approach prevented exclusion of any

potential hits from the initial hit verification process. This threshold was also defined by the capacity for follow up work. For the small molecule screen, a Z-score upper threshold of -2 was used.

2.9 Bacterial culture and plasmid purification

Chemically competent *E. coli*, DH5 α (Invitrogen), were used for all bacterial work. Following transformation, selection of vector-carrying clones was achieved by addition of 100 μ g/ml ampicillin or 50 μ g/ml kanamycin to all media.

Preparation Luria broth (LB) and LB-agar plates

1l of LB was prepared by addition of 25g Luria broth powder to 1l distilled water and subsequent autoclaving. 1l LB-agar (LB-agar) was prepared by dissolving 25g of luria broth powder and 8g of agar powder in 1l of deionised water, followed by autoclaving. LB-agar was melted, cooled, with antibiotic added at this point before being poured into sterile Petri dishes and allowed to cool and set.

Thawing and freezing of bacterial stocks

Before freezing, 5ml of selective LB was inoculated with a single colony or from a glycerol stock and incubated overnight in selective LB at 37°C with

250rpm shaking. 0.67ml aliquots of this culture were then transferred into 1.5ml cryotubes and diluted with 0.33ml sterile 100% glycerol (v/v). Following mixing, aliquots were transferred to -80°C for long-term storage. A sterile loop was used to inoculate a LB culture from frozen glycerol stocks.

Transformation of competent cells

Bacterial transformations were conducted in DH5 α competent cells according to the manufacturers instructions (Invitrogen). Briefly, DH5 α were thawed on ice and 1-10ng DNA added to 50 μ l aliquots. After a 30 minute incubation on ice, the cells were heat-shocked at 42°C for 45 seconds, and then returned to ice for a further 2 minutes. 950 μ l pre-warmed LB media was added to each tube and incubated at 37°C for 1 hour with shaking at 250rpm. 20 μ l and 200 μ l aliquots were spread on pre-warmed selective plates and incubated inverted at 37°C overnight. Individual colonies were picked with a sterile loop and used to inoculate 5ml of selective LB to generate sufficient cells for later use.

Small scale plasmid purification (miniprep)

Plasmid purification from bacterial cultures was performed using Qiagen QIAprep spin miniprep kits according to the manufacturers instructions. Briefly, following overnight growth in 5ml of selective LB in a 50ml tube, bacterial cells were harvested at room temperature by 3 minute centrifugation at 13000rpm. The pellet was re-suspended in 250 μ l P1 buffer and cells lysed by addition of 250 μ l P2 buffer and thorough mixing. Genomic

DNA was precipitated from solution by addition and mixing in of 350 μ l P3 buffer, and the resulting suspension centrifuged at 13,000rpm for 10 minutes at room temperature. The supernatant was pipetted into a QIAprep spin column. The QIAprep column was centrifuged for 1 minute at maximum speed and the flow-through discarded. The column was then washed with 0.5ml PB buffer, centrifuged at 13000rpm for 1 minute and then 0.75ml PE buffer was added before removal by a further centrifugation step as above. Plasmid DNA was eluted by incubating 50 μ l buffer EB on the column for 1 minute before centrifugation into a collection tube.

Medium scale plasmid purification (maxiprep)

Plasmid purification from bacterial cultures was performed using Qiagen QIAprep Spin Maxiprep kits according to the manufacturers instructions. Briefly, a starter culture of 5ml of selective LB was inoculated with a single bacterial colony and grown overnight at 37°C with shaking at 250rpm. 200ml of selective LB was then inoculated with 1ml of starter culture and incubated overnight in a 500ml conical flask at 37°C with shaking at 250rpm. Bacterial cells were harvested by centrifugation at 6000g for 15 minutes at 4°C, and the pellet re-suspended in 10ml P1 buffer. To this suspension 10ml P2 buffer was added, mixed and incubated at room temperature for 5 minutes. Finally, 10ml of chilled P3 buffer was added with mixing and incubated on ice for 20 minutes. Precipitated material was removed by two centrifugation steps at 20000g and 4°C for 30 minutes and 15 minutes, respectively, and the

supernatant applied to a QIAGEN-tip 500 equilibrated with QBT buffer. The tip was washed twice with 30ml QC buffer and eluted with 15ml QF buffer before isopropanol precipitation (0.7 volumes), pelleting of the DNA by centrifugation at 15000g for 20minutes at 4°C and subsequent washing with 70% ethanol. The dried pellet was redissolved in 1ml nuclease-free water and stored at -20 °C.

Large scale plasmid purification (gigaprep)

A starter culture of 5ml selective LB medium was grown overnight at 37°C with shaking at 250rpm following inoculation with a single bacterial colony. The starter culture was then diluted 1/1000 into 2-3L selective LB medium and grown overnight in a 5L conical flask at 37°C with shaking at 250rpm. Following growth, bacterial cells were harvested by centrifugation at 6000g for 15 minutes at 4°C. The bacterial pellet was resuspended in 125ml of P1 buffer, to which 125ml of P2 buffer was added, with thorough mixing followed by a 5 minute incubation at room temperature. 125ml of chilled P3 buffer was then added followed by incubation on ice for 30 minutes. The suspension was centrifuged at 20000g for 30 minutes at 4°C, the supernatant removed and re-centrifuged for 15 minutes. Meanwhile, a QIAGEN-tip 10000 was equilibrated with 75ml QBT buffer and the supernatant then added and allowed to flow-through. The column was then washed with 600ml QC buffer, followed by elution of DNA with 100ml QF buffer. DNA was then precipitated with 70ml room temperature isopropanol and centrifuged immediately at 15000g

for 30 minutes at 4°C. The supernatant was removed and the pellet washed with 10ml of room-temperature 70% ethanol, followed by centrifugation at 15000g for 10 minutes. After removal of the supernatant, the washed DNA pellet was allowed to air-dry for 15 minutes, and subsequently redissolved in 10ml nuclease-free water before aliquoting into 1ml aliquots in 1.5ml tubes and stored at -20 °C.

Following all plasmid purification procedures, DNA concentration was measured using a Nanodrop ND-1000 spectrometer.

2.10 Cloning

All cloning was performed by Dr Csanad Bachrati, Helleday group.

Vectors

With reference to Fig 2.5, a codon optimised expression clone of c9orf102/RAD26L was purchased from Qiagen. The I-SceI plasmid was obtained from M.Jasin, see [165]. A pDsRed-Monomer-C1 (DsRed) plasmid was acquired from Clontech.

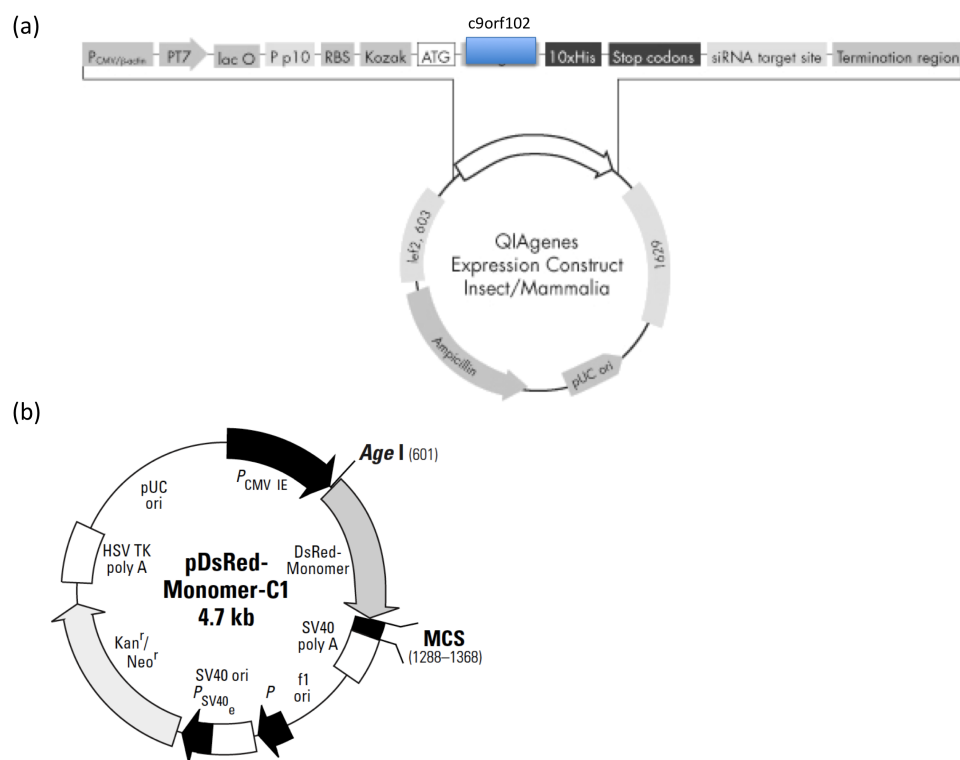


Figure 2.5: Vectors (a) c9orf102 and (b) pDsRed-Monomer-C1.

Chapter 3

Method Development

3.1 The DR-GFP screen

3.1.1 Characterisation of the DR-GFP assay in MRC5 cells

For both the siRNA and small molecule screens, a reproducible, direct and high-throughput homologous recombination assay was required. The assay also had to be reliable; over a period of months-years to facilitate assay development, running of the screens and any follow up work. In order to have as much relevance as possible, preference was given to cell-based assays with human cells being the desired model. Ideally, I wanted to use a primary cell line, to avoid the compromised genetic background of commonly used cancer cell lines influencing the results.

An assay that seemed most appropriate in most of the above regards was

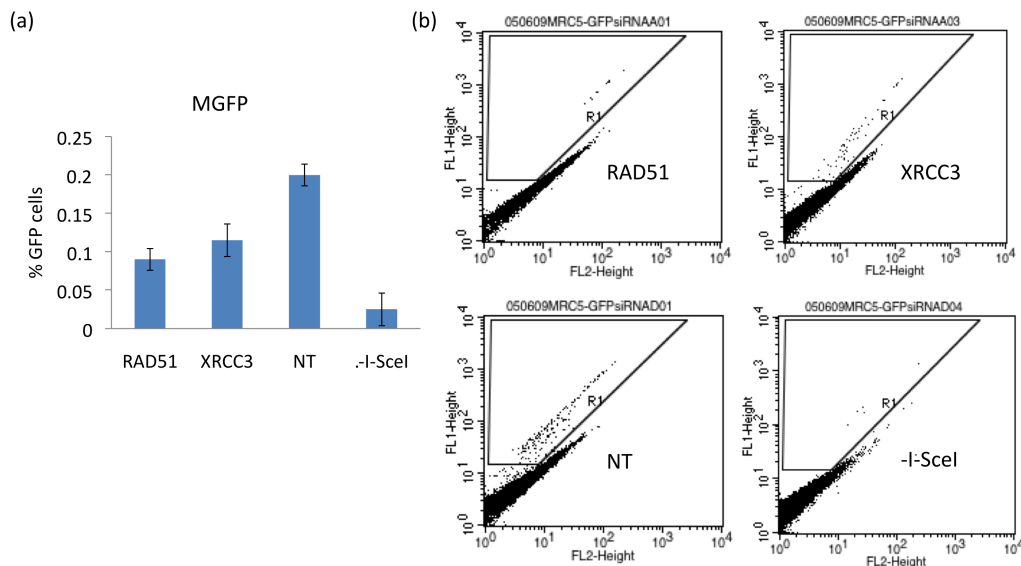


Figure 3.1: Testing of the DR-GFP assay, MGFP cells: **(a)** Initial testing of the siRNA DR-GFP assay in 96-well plate using 50nM siRNA targeting RAD51, XRCC3 and non-targeting (NT) siRNA together with a control in the absence of I-SceI (-I-SceI). siRNA treatment for 48 hours was followed by transfection with I-SceI, with cells isolated for FACS 48 hours following the I-SceI transfection. **(b)** FACS plots of the raw data plotted in (a), showing GFP expressing cells. The Y-axis (FL1) represents green fluorescence, whilst the X-axis (FL2) represents red fluorescence. GFP positive cells lie above the diagonal and are gated in R1. All error bars depicted represent the standard deviation of two replicates.

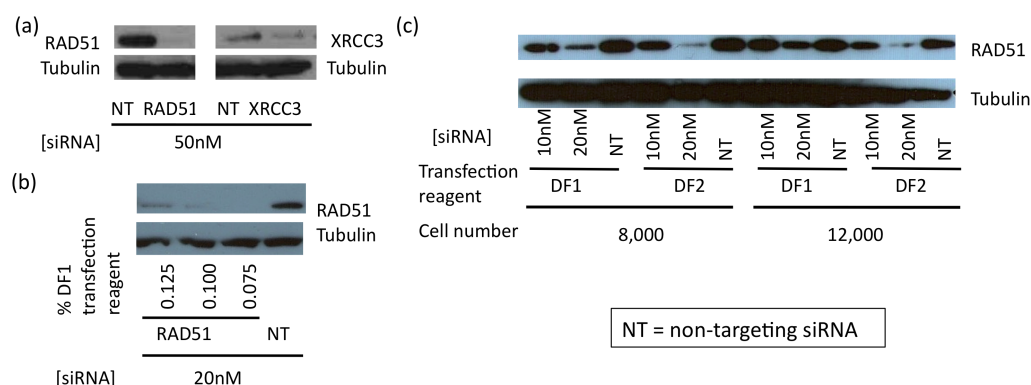


Figure 3.2: Optimisation of the DR-GFP assay in MGFP cells: siRNA mediated protein knockdown optimisation - all Western blots run with samples isolated after 48 hour siRNA treatment. **(a)** Test of siRNA induced knockdown of RAD51 and XRCC3 under those conditions used in Fig 3.1 (a). **(b)** Optimisation of siRNA transfection reagent conditions with respect to RAD51 protein knockdown after a 48 hour siRNA treatment. The transfection reagent DharmaFECT1 (DF1) was used at varying concentrations with a fixed amount (20nM) of siRNA against RAD51 or non-targeting (NT) siRNA. **(c)** Optimisation of siRNA transfection conditions with respect to RAD51 protein knockdown after a 48 hour siRNA treatment. siRNA against RAD51 was tested at 10nM or 20nM in comparison to 20nM NT siRNA. Two different transfection reagents (DharmaFECT1, DF1 of DharmaFECT2, DF2) at 0.075% and two different cell concentrations of 8,000 and 12,000 cells per well were tested in combination.

the DR-GFP assay as developed by the Jasin laboratory [165], see Chapter 2. This cell-based assay may be conducted in human cells and provides an experimental read out that is specific to homologous recombination. The main barrier that needed to be overcome was development of this assay for use in a high-throughput setting. Fortunately, our laboratory possesses a high-throughput FACS machine (BD FACScalibur) that is capable of automated processing of trypsinised cells from a 96-well plate. This setup was used for all the DR-GFP experiments detailed below. With respect to the siRNA screen, all optimisation was performed with a view to using the Qiagen DNA repair and chromatin remodelling siRNA library. This is a focussed library of 579 pools (each pool consisting of four separate siRNAs), targeting the mRNA of a particular DNA repair or chromatin remodelling gene. As detailed in the Materials and Methods Chapter, the siRNA library consisted of four individual siRNAs against each target gene transcript. Each four, unique, oligo-nucleotides against each target transcript were supplied pooled into one well of a 96-well plate and are referred to henceforth as pooled siRNA or siRNA pools. The RAD51 positive control used throughout this study was a pool of 4 separate siRNAs supplied by Qiagen. All siRNA transfections were performed using the reverse transfection protocol (Materials and Methods) where trypsinised, suspended cells are plated into wells of a 96-well plate that already contain the siRNA-transfection reagent mix. This protocol reduces the time required for a siRNA transfection as compared to a forward transfection where transfection reagent is added to pre-plated cells.

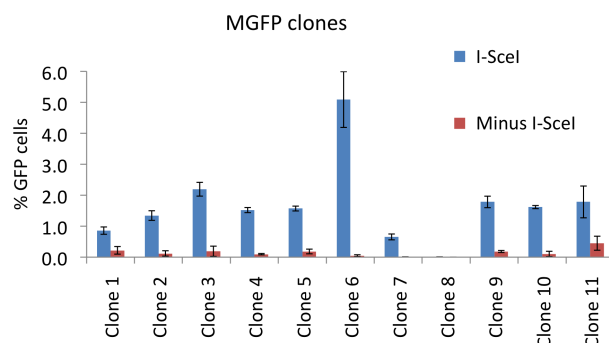


Figure 3.3: Testing of clones derived from the MGFP cell line in the DR-GFP assay, I-SceI transfection was conducted 24 hours following the plating of cells and cells isolated for FACS 48 hours after I-SceI transfection (or transfection reagent only treatment). Error bars depicted represent the standard deviation of two replicates.

All initial experimental development was performed in MRC5-DR-GFP (MGFP) cells with respect to the siRNA screen. The methodology was later adapted for subsequent small molecule work. Of initial importance was to test the assay in a 96-well setting using my desired protocol. I elected to establish siRNA knockdown by reverse siRNA transfection for 48 hours, before introduction of a site-specific double strand break in the DR-GFP reporter via transfection of the I-SceI endonuclease. Results were then processed by FACS 48 hours after the I-SceI transfection. It was important, at this stage, to test a positive siRNA control for these assays. To this end, siRNAs targeting both RAD51 and XRCC3 were used together with a non-targeting (NT) siRNA negative control.

Initial tests of the siRNA protocol in MGFP cells (Fig 3.1) demonstrated that the assay was functional, with an I-SceI specific induction of GFP fluorescence as measured by FACS at 96 hours after siRNA transfection, i.e. with 48 hours to establish protein knockdown, then 48 hours to induce GFP expression following I-SceI expression. Both the RAD51 and XRCC3 siRNAs gave a significant reduction in the percentage of GFP positive cells, suggesting that either would make a suitable siRNA positive control. Knockdown of both RAD51 and XRCC3 was verified by Western blot 48 hours following reverse transfection of 8000 cells (Fig 3.2). These data suggested that knockdown of proteins required for HR, before induction of a site-specific double strand break in the DR-GFP assay, was sufficient to reduce HR efficiency as measured by the percentage of GFP positive cells in the DR-GFP assay in my hands. However, it was evident at this stage that the assay required optimisation. Firstly, the level of GFP expressing cells, even after NT siRNA treatment, was very low (under 0.25%). This problem was exacerbated by the modest phenotype displayed by both RAD51 and XRCC3 knockdown, leaving a small dynamic range in which to detect hits in the proposed siRNA screen. A solution to these problems was pursued by the optimisation of siRNA transfections with regard to the transfection reagent and siRNA concentration (Fig 3.2). This allowed the establishment of conditions which gave efficient protein knockdown of the RAD51 positive control, specifically 0.075% DharmaFECT2 (DF2) and 20nM siRNA. Cell number at the point of reverse transfection did not have a significant effect. All I-SceI transfec-

tions were conducted under standard conditions, which had previously been optimised in our lab (Table 2.5).

A possible explanation for the low induction of GFP positive cells by I-SceI transfection seen in the MGFP cells was their heterogeneity. To circumvent this drawback, clones of MGFP cells were isolated and selected for testing under standard I-SceI transfection conditions. This allowed identification of a clone (clone 6, or MGFP6) that gave a much higher induction of GFP expression following site-specific double strand break induction with I-SceI (Fig 3.3). The MGFP6 cells were used for subsequent method development.

3.1.2 Method development in MGFP6 cells

With the MGFP6 cells selected for screen development, I continued the siRNA knockdown optimisation. In order to increase confidence in the proposed screen, I established that the siRNA transfection conditions defined above were effective over an extended period of time (up to 96 hours following reverse transfection - the endpoint of the assay), at different siRNA concentrations and against multiple protein targets (Fig 3.4 (a)). I found that the protocol was effective at all timepoints and even at the lower siRNA concentration of 10nM. These conditions (10nM siRNA, 0.075% DF2) were also tested successfully using the robotic handling station and the appropriately modified transfection protocol (see Figs 2.2, 3.4).

Having determined the effectiveness of the transfection protocol, the DR-

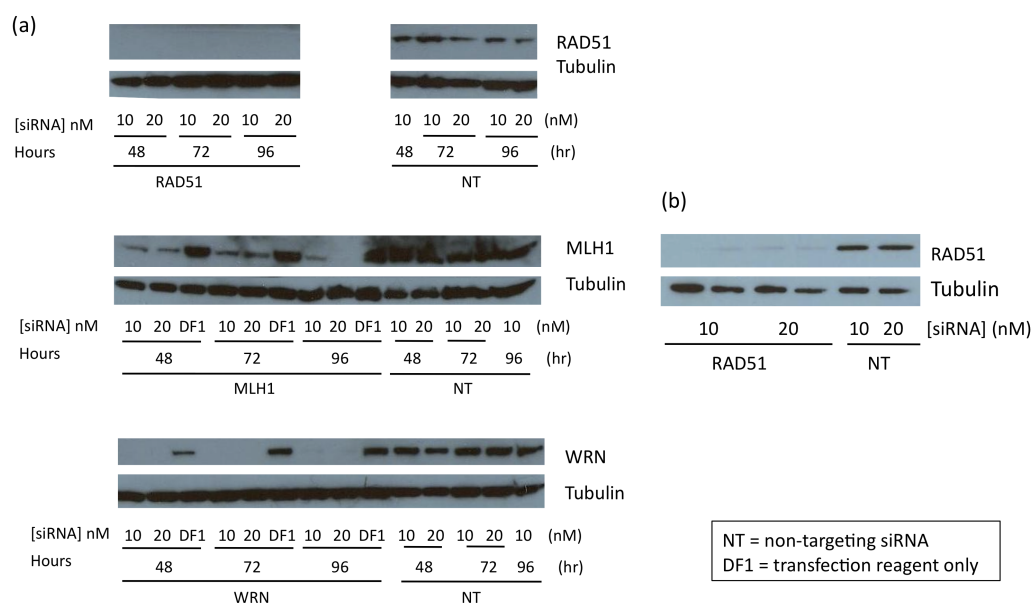


Figure 3.4: Optimisation of siRNA transfection in MGFP6 cells: **(a)** Protein knockdown at different timepoints (post siRNA transfection), at different siRNA concentrations and against different targets (RAD51, MLH1 and WRN). Knockdown levels are compared to both NT (non-targeting siRNA) and DF1 (transfection reagent only) controls. **(b)** Confirmation of protein knockdown with the robotic siRNA transfection protocol, performed at 10nM and 20nM siRNA concentrations. Antibody details are provided in Table 2.6.

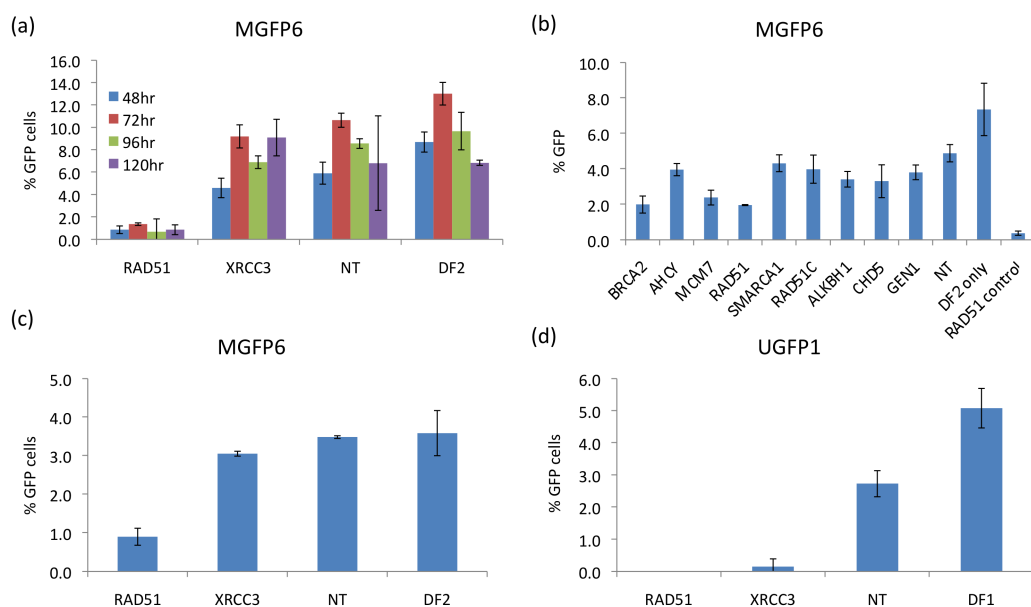


Figure 3.5: Characterisation of the DR-GFP assay in MGFP6 and UGFP1 cells: **(a)** DR-GFP assay test in MGFP6 cells with multiple timepoints taken following I-SceI transfection. **(b)** Test of multiple siRNAs from the Qiagen siRNA library in the DR-GFP assay in MGFP6 cells at 48 hours post I-SceI transfection. **(c)** and **(d)** contrast of the DR-GFP assay conducted side by side in both MGFP6 and UGFP1 (U2OS-DR-GFP clone 1) cell lines following siRNA or blank (DF1 or DF2) treatment before I-SceI transfection (48 hours). All error bars depicted represent the standard deviation of two replicates.

GFP assay was tested in 96-well plates: 48 hours following siRNA reverse transfection, I-SceI transfection was performed and cells were analysed for GFP induction from 48-120 hours after this point (Fig 3.5 (a)). GFP expression peaked 72 hours after I-SceI transfection, however as this would be 120 hours after siRNA treatment (allowing for possible recovery of protein expression) and make for a six day assay, I decided to harvest cells for FACS 48 hours after I-SceI treatment, marking out a compromise between efficient protein knockdown and maximum GFP induction. The final assay conditions I elected upon were as follows; establishment of protein knockdown by siRNA treatment for 48 hours followed by induction of GFP expression by I-SceI transfection. FACS was conducted 48 hours after I-SceI transfection.

It was noted at this point that the phenotype demonstrated from XRCC3 knockdown was not significantly different from NT siRNA treated cells and this extended the concern that it would prove difficult to identify siRNAs that led to a significant, but not dramatic (as in the case of RAD51 siRNA) inhibition of HR. To further investigate this problem, a number of different siRNAs taken from the Qiagen siRNA library (including RAD51), along with the Qiagen RAD51 control (identical to the Qiagen library RAD51 siRNA), were tested in the DR-GFP assay with the conditions defined from the previous work. These siRNAs targeted both known HR proteins and non-HR proteins with a view to gaining an idea of the kind of data distribution I might achieve when it came to running the screen (Fig 3.5 (b)). These data made it clear that the DR-GFP assay in the MGFP6 cells was unlikely to

be capable of producing a suitable range between the positive and negative siRNA controls (RAD51 and NT, respectively) in which to characterise a large number of siRNAs with respect to their effects on HR. Of particular note was the limited phenotype exhibited by the BRCA2 siRNA (Fig 3.5 (b)).

3.1.3 Method development in UGFP1 cells

At this point I decided to test the DR-GFP assay in the UGFP1 cell line which had been prepared by Dr Cecilia Lundin (Helleday lab) by stable transfection of U2OS cells with the DR-GFP vector and subsequent clonal selection as outlined above for the MGFP positive cells. siRNA and I-SceI transfection conditions had been defined by her in a similar process to my work with the MGFP positive cells. This work had demonstrated that DharmaFECT 1 (DF1) was the optimum transfection reagent for work in U2OS cells and accordingly it was adopted for use in all subsequent siRNA experiments in U2OS cells. Fig 3.5 (c) shows a comparison, conducted by the author, of the DR-GFP assay run side by side in MGFP6 and UGFP1 cells. It is immediately apparent from this experiment that the UGFP1 cells are more sensitive to RAD51 and XRCC3 knockdown in contrast to the MGFP6 cells, although it was also noted that the NT siRNA gave a reduction in % GFP positive cells relative to DF1 only treated cells. In light of these favourable data, demonstrating the increased dynamic range of the DR-GFP assay in UGFP1 cells, I opted to develop the UGFP1 cells for the siRNA

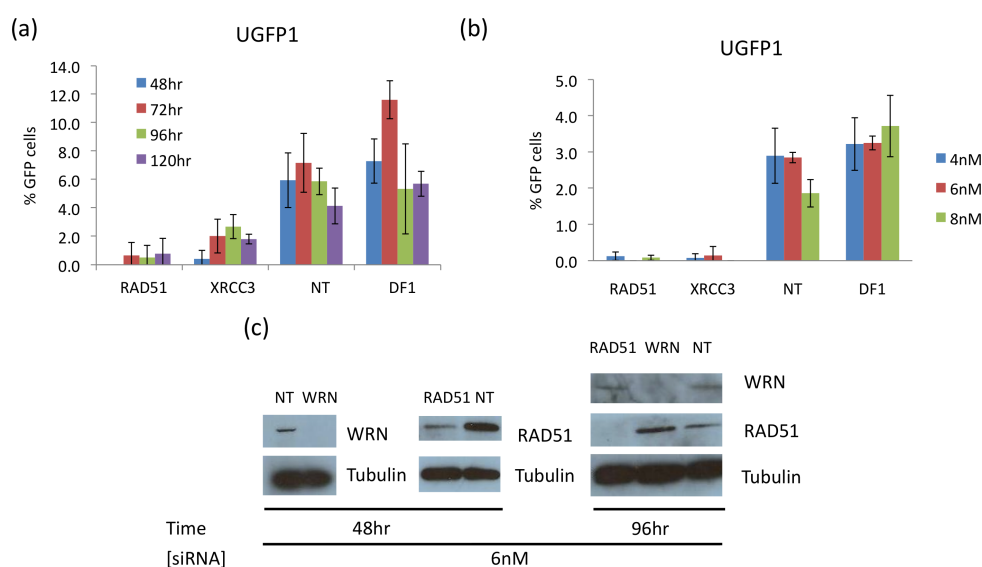


Figure 3.6: Characterisation of the DR-GFP assay in UGFP1 cells: **(a)** DR-GFP assay test with 10nM siRNA in UGFP1 cells with multiple timepoints taken following I-SceI transfection as indicated in the chart legend. **(b)** DR-GFP assay test with 4-, 6- and 8nM siRNA with FACS performed 48 hours after I-SceI transfection. **(c)** Knockdown of both RAD51 and WRN at both 48- and 96 hours following reverse transfection with 6nM of siRNA. All error bars depicted represent the standard deviation of two replicates.

screen.

In an identical approach to my optimisation of the MGFP6 cells for the siRNA screen, I tested the DR-GFP assay in UGFP1 cells (Fig 3.6 (a)) with varying timepoints following I-SceI transfection, (siRNA and I-SceI transfection conditions had previously been optimised by C.Lundin). In line with earlier observations in MGFP6 cells, GFP induction peaked around 72 hours following I-SceI transfection. However, a comparable induction was still ap-

parent after 48 hours and for the reasons outlined above for the MGFP6 cells, this endpoint was selected for the screen. At this point, it was noted that the parameters used for high-throughput sample reading [which had been used for the DR-GFP timepoint experiments in Figs 3.5 (a) and 3.6 (a) and (b)] were giving rise to large differences between replicates. Subsequent experiments, including the screen itself, were run with larger sample volumes and a slower sample read time, maximising cell pick up efficiency by the FACS machine whilst I decided to perform the screen itself and any follow up work in triplicate.

A remaining optimisation step was to test an internal control for I-SceI transfection efficiency, with a view to normalising all data in the screen with respect to this information. To this end, I cotransfected the I-SceI plasmid together with an RFP encoding plasmid (DsRed). This cotransfection was performed across a 96-well plate and is visualised using a heat map of the plate in Fig 3.7. Whilst it was apparent that the level of GFP expressing cells generally correlated with that of the RFP expressing cells across all wells, I did not feel that the correlation was of high enough quality to use for data normalisation. Also of note from these data, was the inherent well to well variability in GFP expression, which must be borne in mind when considering the screen data.

The siRNA screen was now run in the UGFP1 cells, by robot, with 10nM siRNA (see Chapter 4). 10nM siRNA was chosen in order to maximise protein knockdown, despite the concerns outlined above. It became apparent

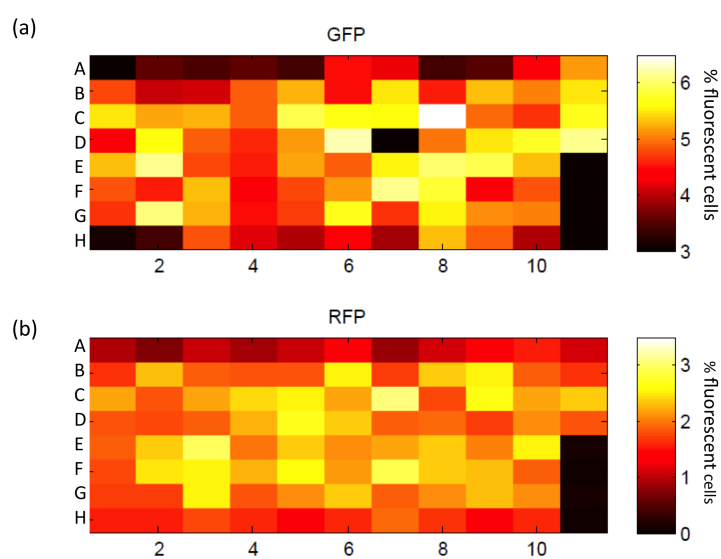


Figure 3.7: 96-well plate heat map following I-SceI and dsRed cotransfection of UGFP1 cells (48 hours). **(a)** % GFP positive cells and **(b)** % RFP cells as measured by FACS. Scale bars are shown on the right hand side.

upon analysis of the screen data that the previously observed decrease of the percentage of GFP expressing cells following NT siRNA treatment relative to DF1 only treated cells was consistent across the screen, although there was no toxicity induced by NT siRNA treatment. This possible off-target effect, coupled with a modest induction of GFP expression led me to test whether the siRNA concentration used for screening could be decreased to reduce the observed, non-specific siRNA mediated effect and thus increase the dynamic range of the assay (Fig 3.6 (b)). This was successful. Upon comparison of the DR-GFP test data with 4, 6 and 8nM siRNA, it was evident that reduction of the siRNA concentration to 6nM eliminated the effect of the NT siRNA whilst retaining the phenotype produced by both RAD51 and XRCC3 knock-down. Confidence in the effectiveness of the siRNA transfection protocol was bolstered by the favourable results observed in the DR-GFP assay with as little as 4nM of siRNA. In order to validate the DR-GFP data, protein knock-downs with 6nM siRNA for both RAD51 and WRN were demonstrated at both 48- and 96- hours after reverse transfection (Fig 3.6). The siRNA screen was repeated using these refined assay conditions. Each siRNA screen was run in two batches of four siRNA plates (corresponding to 12 plates when performed in triplicate) per run.

3.2 Small molecule screen assay development

As has been outlined above, optimisation work for the siRNA screen provided the knowledge base for development of the DR-GFP assay for the small

molecule screen. However, this is not the only screening approach that I considered. Although the DR-GFP assay has a number of advantages and can be used in 96-well format as detailed above, it lacks the throughput capacity for screening 1000s of compounds in a timely manner in a small molecule screen. The rate limiting step of the DR-GFP assay is reading the plates on the FACS machine, which takes around one hour per plate. This was feasible for the relatively small scale siRNA library but not ideal for a small molecule screen, numbering thousands of compounds. It was therefore necessary to consider an alternative screening approach.

3.2.1 Synthetic lethal screen

It has been well documented that HR deficient cells are exquisitely sensitive to PARP inhibitors (see Chapter 1). I proposed to exploit and reverse this synthetic lethal condition by using PARP1 knockout mouse embryonic fibroblasts (MEFs) to screen for HR inhibitors. The hypothesis was that HR inhibitors would prove synthetic lethal to the PARP1 knockout MEFs. By screening compounds against both PARP1^{-/-} (A11) MEFs and their isogenic PARP^{wt} (A19) MEFs in parallel, I proposed that compounds specifically toxic to the A11 MEFs were likely to be HR inhibitors.

As with any screen, a positive control was required i.e. an HR inhibitor. Although there is a dearth of specific HR inhibitors, a number of small molecules designed to target non-HR proteins have been shown to inhibit HR (see Chapter 1). I opted to use the histone deacetylase inhibitor, PCI-

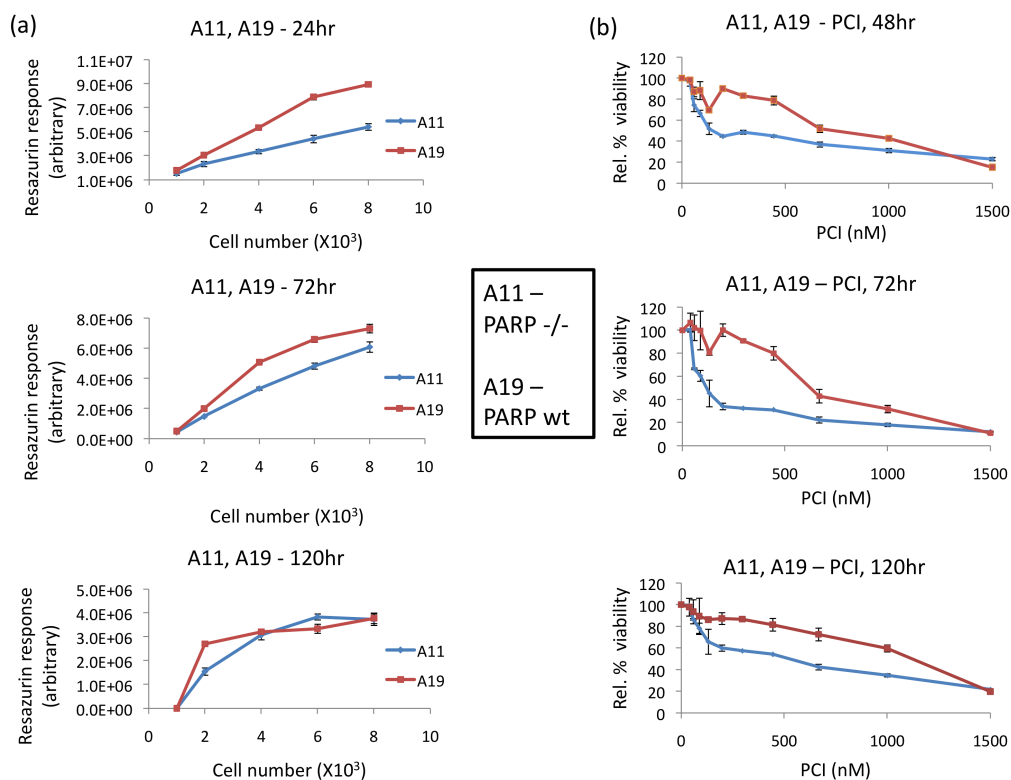


Figure 3.8: Characterisation of A11, PARP ^{-/-} and A19, PARP wt MEFs: **(a)** Resazurin cell viability assay response (arbitrary fluorescence units) for A11 and A19 MEFs seeded at 1-,2-,4-,6- and 8- thousand cells per well and measured at 24-,72- and 120 hours after seeding. **(b)** Relative cell viability curves for A11 and A19 MEFs following PCI treatment over a range of doses at 48-,72- and 120 hours following PCI treatment (all data points normalised to the DMSO control). A11 shown in blue, A19 in red. All error bars depicted represent the standard deviation of two replicates.

24781 (PCI) for my small molecule screen positive control. This compound has been shown to reduce RAD51 expression levels and accordingly HR activity.

Initial work characterised the growth properties of the low passage number A11 and A19 MEFs (passages 4 and 11 respectively) with respect to both cell seeding density and time post seeding. It was important to define seeding conditions that allowed use of the viability assay within its linear range over multiple timepoints. Fig 3.8 (a) shows the Resazurin (cell viability assay) response for both A11 and A19 MEFs seeded at different cell concentrations and measured at different timepoints post-seeding. These data demonstrate that seeding of A11 MEFs at two thousand cells per well and A19 MEFs four thousand cells per well provided cell growth within the linear range across the measured time points. Furthermore, treatment with PCI was demonstrated to lead to the selective killing of A11 cells (Fig 3.8 (b)).

These data provided the confidence to proceed to running a test screen using the robot. A PCI concentration of 500nM was selected as a positive control due to the observed selective toxicity for A11 MEFs at this dose (Fig 3.8 (b)). Accordingly, 24 hours after seeding, cells were treated with PCI or DMSO. Fig 3.9 (a) shows the unexpected results obtained from this test, with the PCI mediated toxicity almost equal in both A11 and A19 MEFs, contrary to the effects observed in Fig 3.8 (b). This surprising result led me to test the stability of the phenotype observed in Fig 3.8 (b). Surprisingly, I witnessed a cell passage number dependent diminution of the initially

observed PCI mediated specific killing of A11 MEFs (Fig 3.9 (b)). This phenotypic instability is difficult to account for, but may result from either a longer recovery time after thawing for the A11 cells, making them especially sensitive to drug treatments or from their instability resulting from PARP1 knockout providing a changing phenotype with time. Whatever the cause, it was clear, at this stage, that this assay was not appropriate for the proposed small molecule screen.

3.2.2 DR-GFP small molecule screen

With the synthetic lethal screening approach ruled out and having demonstrated that the DR-GFP assay in UGFP1 cells was suitable for high-throughput screening, albeit with questions over throughput, I adapted the assay for small molecule screening using the protocol outlined in Fig 2.2. 24 hours following seeding, the UGFP1 cells were treated with a titration of PCI before immediate I-SceI transfection. This treatment regime is referred to in relation to the small molecule screen as a 'cotreatment'. Results from the DR-GFP and cell viability assays (Fig 3.10 (a) and (b)) demonstrate that PCI is capable of inhibiting the DR-GFP assay in UGFP1 cells and that this inhibitory activity is not driven by toxicity. It was decided to use PCI at both 400nM and 600nM in the small molecule screen, to be performed in triplicate. 400nM PCI was shown to reduce RAD51 protein levels following a 24 hour treatment (Fig 3.10 (c)), consistent with both the published data (see Chapter 1) and its inhibitory effect seen in the DR-GFP assay. To

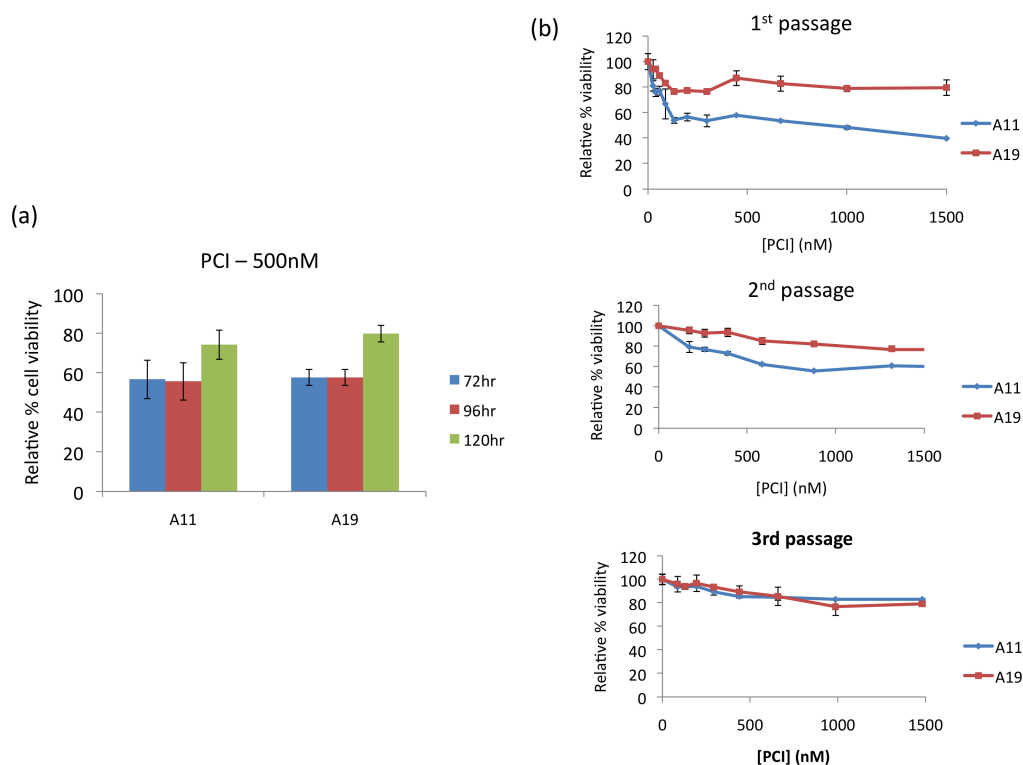


Figure 3.9: Further testing of A11 and A19 MEFs: **(a)** Relative % cell viability data (normalised to DMSO treated cells) for a test screen run in 96-well plate using the robot in the A11 and A19 MEFs with PCI at 500nM used as the positive control. **(b)** PCI relative cell viability (DMSO normalised) dose responses for A11 and A19 MEFs with respect to passage number. A11 shown in blue, A19 in red. All error bars depicted represent the standard deviation of two replicates.

address the issue of throughput for the proposed small molecule screen, I conducted a test optimisation of the small molecule cotreatment protocol in the DR-GFP assay in a 384-well plate (Fig 3.10 (d)). The 384-well plate protocol yielded excessively large errors between replicates at all cell densities used, rendering this approach inappropriate for the small molecule screen. The principle source of the errors was the low cell number, compounded by the inefficient pick up of cells from the 384-well plate by the FACS machine. Accordingly, I opted to use the DR-GFP assay in UGFP1 cells conducted in 96-well plates for the small molecule screen.

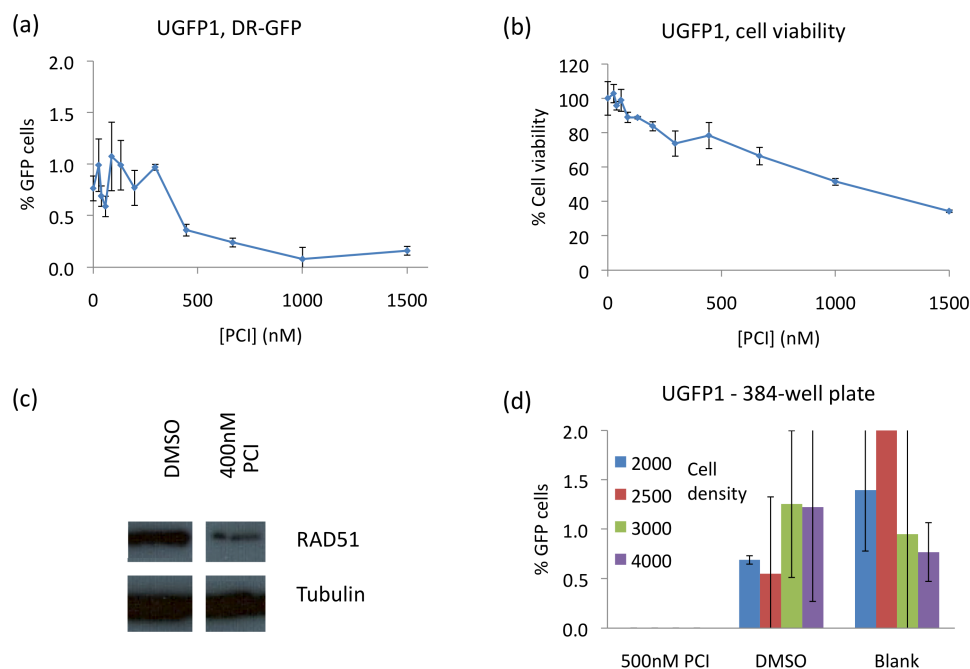


Figure 3.10: Characterisation of UGFP1 cells for small molecule screen: Dose response assays with PCI were conducted in both **(a)** the DR-GFP assay performed in accordance with the Materials and Methods with cells seeded at 12,000 per well and **(b)** the cell viability assay with cells seeded at 12,000 per well. **(c)** The effect of 24 hour PCI treatment upon RAD51 expression levels in UGFP1 cells (12,000 per well) was also tested by Western blot. (a)-(c) were all conducted in 96 well plate. **(d)** The small molecule screen assay was tested in 384-well plate in the DR-GFP assay (UGFP1 cells). The positive control, PCI, was tested at 500nM. Four different cell densities were tested across all conditions; cell number is shown in the figure legend. All error bars depicted represent the standard deviation of two replicates.

Chapter 4

Results: siRNA screen

4.1 siRNA screens

4.1.1 10nM screen

Following optimisation of the UGFP1 cells for the high-throughput DR-GFP assay, the siRNA screen was run at an siRNA concentration of 10nM, which had been demonstrated to provide robust protein knockdown and a reasonable dynamic range for data collection (Fig 3.5). Initial analysis of the screen data, including quantification of the controls for each replicate plate (Fig 4.1 (a)) and replicate analysis (Fig 4.1 (b)), highlights a number of potential problems with the 10nM screen, which needed to be addressed (see below). Firstly, the overall induction of GFP was low across most of the plates and although relative values were consistent from plate to plate, absolute % GFP positive cell values were variable. The NT siRNA treated control wells some-

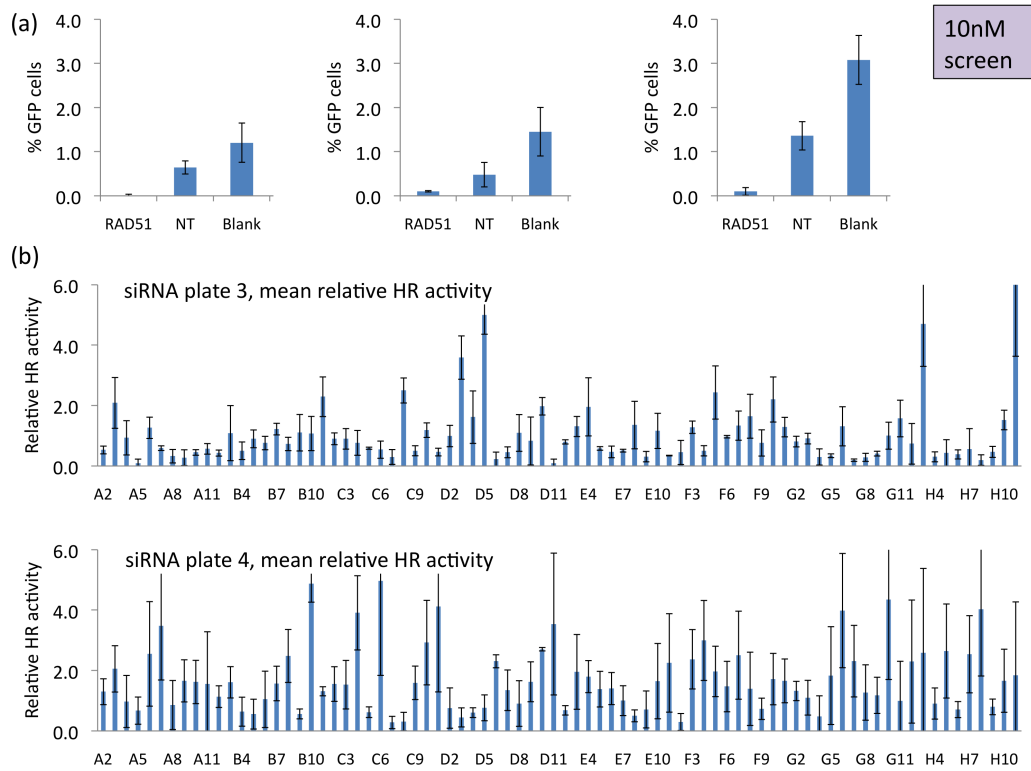


Figure 4.1: siRNA screen in UGFP1 cells at 10nM. **(a)** Representative DR-GFP assay data for control wells of three individual replicate plates from the 10nM siRNA screen (24 plates in total). Each siRNA library plate (8 in total) was tested in triplicate. **(b)** Representative DR-GFP assay data from two of the eight siRNA library plates, comprising the mean of three replicates, each normalised to the NT siRNA control. Each well represents an siRNA pool targeting a particular gene. All error bars depicted represent the standard deviation of at least two replicates. In some cases replicates were excluded where the FACS machine had failed to pick up sufficient cells for counting.

times displayed below 1% GFP positive cells. It was also clear that the previously observed phenotype from the NT siRNA, as compared to the blank (DF1 only treated) cells was still present. This off target effect of the NT siRNA, coupled to the low level of GFP induction and sizeable differences between replicates served to minimise the dynamic range of the assay and reduce confidence in the results and is what led to the testing of lower siRNA concentrations for the DR-GFP assay 3.6. Nonetheless, examination of the normalised HR activity for each siRNA plate (Fig 4.1 (b)), revealed that those siRNAs causing a decrease of HR activity often replicated consistently across the screen. There were also a relatively large number of siRNAs which led to an increase in relative HR activity. This may be accounted for by the effect of the NT siRNA to which all points of the dataset were normalised.

4.1.2 6nM screen

Realising that the 10nM screen had some clear drawbacks and following re-optimisation of siRNA knockdown conditions (Chapter 3), I re-ran the siRNA screen at an siRNA concentration of 6nM, with freshly thawed and recovered UGFP1 cells aiming both to reduce the off-target effect of the NT siRNA, whilst increasing levels of GFP induction. Analysis of the 6nM screen data revealed some key improvements (Fig 4.2). The off-target effect of the NT siRNA treated cells was diminished (albeit not eliminated) at the lower siRNA concentration and the level of GFP induction was also increased. These changes improved the quality of the screen dataset. It

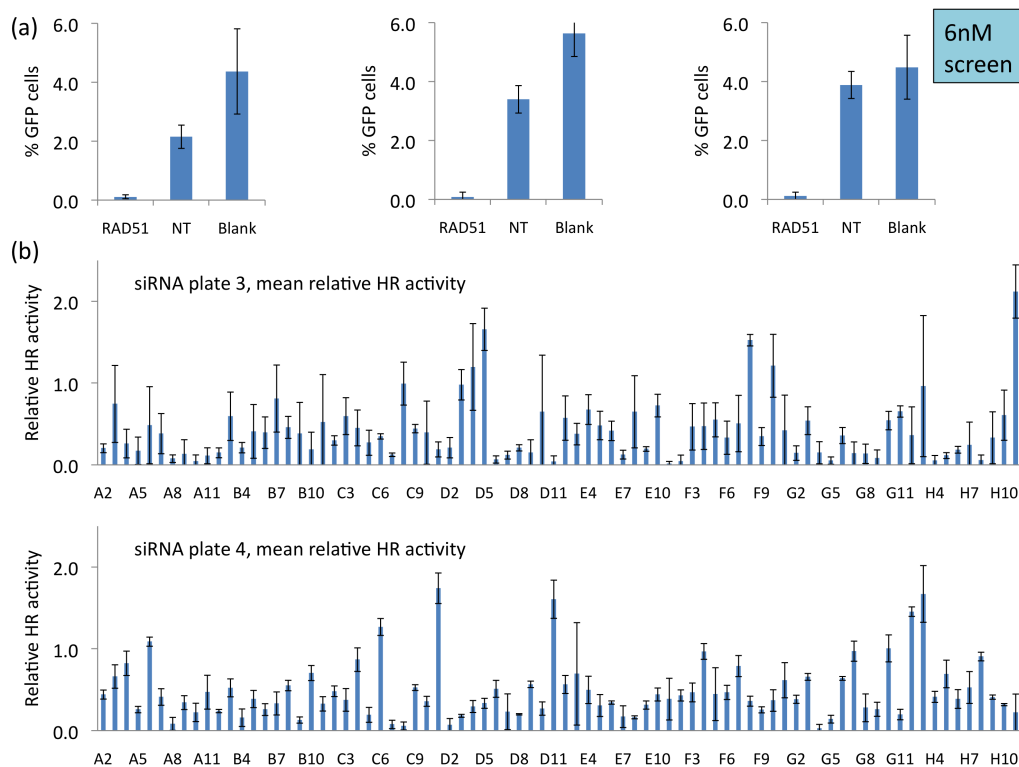


Figure 4.2: siRNA screen in UGFP1 cells at 6nM. **(a)** Representative DR-GFP assay data for control wells of three individual replicate plates from the 6nM siRNA screen (24 plates in total). Each siRNA library plate (8 in total) was tested in triplicate. **(b)** Representative DR-GFP assay data from two of the eight siRNA library plates, comprising the mean of three replicates, each normalised to the NT siRNA control. Each well represents an siRNA pool targeting a particular gene. All error bars depicted represent the standard deviation of at least two replicates. In some cases replicates were excluded where the FACS machine had failed to pick up sufficient cells for counting.

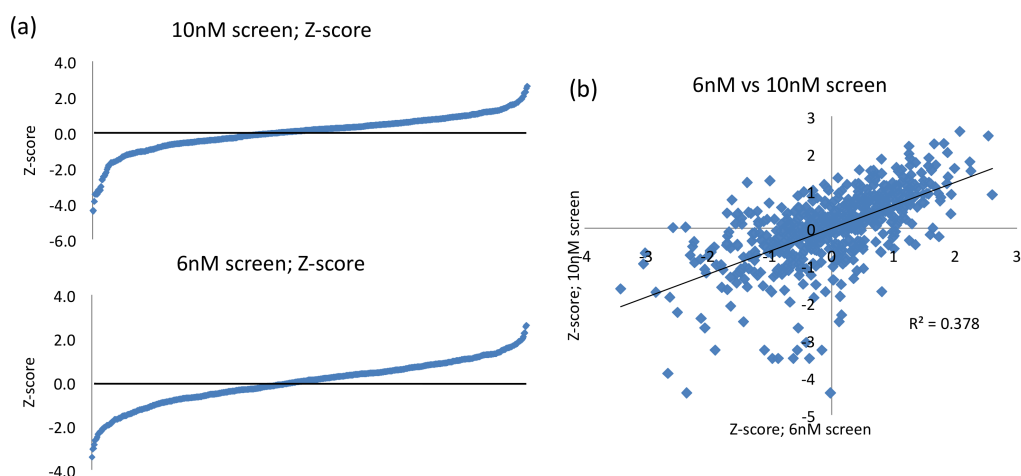


Figure 4.3: siRNA screen datasets. **(a)** Ranked Z-scores for the 10nM and 6nM siRNA screens in the UGFP1 cells. Each data point represents the Z-score calculated from the triplicate data obtained for one siRNA pool from the siRNA library. **(b)** Correlation plot of the 10nM screen Z-scores versus the 6nM screen Z-scores.

was also observed that across both screens, the RAD51 siRNA control was consistently effective.

A profile of the complete Z-score dataset for each screen, together with a correlation plot between the two datasets, is shown in Fig 4.3. The ranked Z-score data demonstrates a normal distribution across each screen, whilst the correlation plot may reflect both the change in conditions between the two screens as well as the inherent variability of this assay in a high-throughput setting.

10nM screen			6nM screen		
	Target	Z-score		Target	Z-score
1	COPB2	-4.4		UBA52	-3.4
2	NUP205	-4.4		SNRPF	-3.0
3	BRCA2	-3.9		ATRIP	-3.0
4	JMJD1B	-3.5		BRCA1	-2.8
5	CHD8	-3.5		BRCA2	-2.6
6	FANCM	-3.5		PALB2	-2.6
7	XAB2	-3.5		POLL	-2.6
8	C17orf101	-3.3		MAD1L1	-2.5
9	BTBD12 / SLX4	-3.3		TRIM28	-2.4
10	MYST1 / hMOF	-3.3	<-2.0	COPB2	-2.3
11	EME2	-3.3		HIF1AN	-2.3
12	C9orf102	-3.1		RPL35	-2.2
13	PSMC4	-2.7		MBD1	-2.2
14	SNRPE	-2.7		SUPV3L1	-2.2
15	ASPH	-2.5		MUTYH	-2.1
16	NTHL1	-2.5		SHFM1/DSS1	-2.1
17	SHFM1 / DSS1	-2.4		MTHFD2	-2.1
18	TAL1	-2.3		SNRPE	-2.0
19	MAD1L1	-2.3		NUDT7	-2.0
20	TREX1	-2.1		HIST2H2AA3	-2.0
21	MSH4	-2.0		PLK1	-2.0
22	NUDT1	-1.9		IRS1	-2.0
23	PALB2	-1.9		SMARCA1	-1.9
24	IFNGR2	-1.8		RAD18	-1.9
25	BRCA1	-1.7		EP400	-1.9
26	TGIF1	-1.7		EME2	-1.9
27	FANCG	-1.7		CCND1	-1.9
28	TOPBP1	-1.7	<-1.5	RAD9A	-1.8
29	PAICS	-1.7		ORC2L	-1.8
30	REV3L	-1.7		PSMA1	-1.8
31	RPA1	-1.6		ALKBH2	-1.7
32	UBA52	-1.6		IGF2	-1.7
33	ORC2L	-1.6		PAFAH1B3	-1.7
34	NBN / NBS1	-1.6		RPA1	-1.7
35				POT1	-1.7
36				SETD8	-1.7
37				APEX1	-1.6
38				ERCC2	-1.6
39				TREX1	-1.6
40				HMGB2	-1.6
41				TET3	-1.6

Figure 4.4: siRNA screen hit-list showing Z-score values for both the 10nM and 6nM screens. Known DSB repair proteins are highlighted in orange and proteasome subunits in blue.

4.1.3 siRNA screen - hits

With the siRNA screening now complete, it was important to define a hit list for further work. Although there were some limitations with the 10nM screen data, both the 6nM and 10nM datasets were processed using the Z-score metric (Chapter 2), providing values representing the distance of each point of the dataset from the the mean of the population. All Z-scores under 0 represent a decrease in the percentage of GFP positive cells relative to the population mean. A Z-score of -1 is a datapoint one standard deviation below the mean, -2 is two standard deviations below the mean and so on. Z-score analysis allowed ranking of the hits with respect to their statistical significance (but not necessarily the magnitude of their effect in the DR-GFP assay). A standard cut off for a statistically significant hit in a biological screen of this nature is a Z-score of under -2 (i.e. any datapoint lying greater than two standard deviations below the mean). Analysis of both data sets (Fig 4.4) showed this cut-off yielded 21 and 22 hits in the 10nM and 6nM screens, respectively, or around 3.5% of the siRNA library. The hits identified in each separate screen showed only a limited amount of overlap but in both cases, a number of siRNAs targeting known DSB repair genes were identified under the initial -2 cut-off (Fig 4.4) DSB repair genes highlighted in yellow). These included the key HR gene BRCA2 and its binding partner DSS1 in both screens. Of additional interest, given the documented effect of proteasome inhibitors on HR was the presence in the hit-list of siRNAs targeting proteasome subunits (Fig 4.4 highlighted in blue). It is also note-

worthy that numerous repair factors from non-HR pathways, in particular NER and BER were identified in both screens (Fig 4.4 and 4.1). This result raises the possibility of interplay between HR and other repair pathways not traditionally associated with DSB repair.

Table 4.1: siRNA screen hit-list compiled from the 10nM and 6nM screens.

Target	Z-score	function
ALKBH2	-1.7	E.coli nucleic acid demethylase alkB, homolog 2
APEX1	-1.6	BER, AP-endonuclease
ASPH	-2.5	Aspartate beta-hydroxylase
ATRIP	-3.0	ATR-interacting protein
BRCA1	-2.8	Multiple DSBR pathways
BRCA2	-3.9	Multiple DSBR pathways
BRD1	-1.4	Bromodomain containing 1 - histone acetyl lysine binding
BTBD12 / SLX4	-3.3	Structure specific endonuclease complex member
C17orf101	-3.3	Uncharacterised
C9orf102	-3.1	Uncharacterised
CCND1	-1.9	Cyclin D1
CHD8	-3.5	Chromodomain helicase DNA binding protein 8
COPB2	-4.4	Coatomer protein complex member - Golgi trafficking
EME2/SLS2	-3.3	Repair endonuclease - not characterised

EP400	-1.9	E1A binding protein
ERCC2	-1.6	NER, XPD helicase
FANCG	-1.7	Multiple DSB β R pathways
FANCM	-3.5	Multiple DSB β R pathways
HIF1AN	-2.3	Hypoxia inducible factor 1
HIST2H2AA3	-2.0	Histone H2A family member
HMGB2	-1.6	High-mobility group box 2
IFNGR2	-1.8	Interferon gamma receptor 2
IGF2	-1.7	Insulin-like growth factor 2
IRS1	-2.0	Insulin receptor substrate
KDM3B/JMJD1B	-3.5	Lysine demethylase 3
MAD1L1	-2.5	Spindle checkpoint protein
MBD1	-2.2	Methyl-CpG binding domain protein 1
MSH4	-2.0	MutS homologue 4
MTHFD2	-2.1	Mitochondrial metabolism
MUTYH	-2.1	MMR - MutY homologue
MYST1 / hMOF	-3.3	Histone acetyl transferase
NBN / NBS1	-1.6	MRN complex member
NTHL1	-2.5	BER endonuclease
NUDT1	-1.9	Hydrolysis of damaged nucleotide bases
NUP205	-4.4	Nucleoporin
ORC2L	-1.8	Origin recognition complex protein
PAFAH1B3	-1.7	Platelet-activating factor acetylhydrolase subunit

PAICS/ADE2	-1.7	Purine biosynthesis
PALB2	-1.9	Partner and localizer of BRCA2
PLK1	-2.0	Polo-like kinase 1 - G2-M checkpoint
POLL	-2.6	Pol lambda
POT1	-1.7	Protection of telomeres
PSMA1	-1.8	Proteasome subunit
PSMC4	-2.7	Proteasome subunit
RAD18	-1.9	E3 ubiquitin ligase, targets PCNA in response to DNA damage
RAD9A	-1.8	9-1-1 complex
REV3L	-1.7	Catalytic subunit of DNA pol zeta
RPA1	-1.6	Coats resected ssDNA
RPL35	-2.2	RPA 30kDa subunit
SETD8	-1.7	Ribosomal protein L35
SHFM1/DSS1	-2.1	BRCA2 binding partner
SMARCA1	-1.9	SWI/SNF related DNA translocase
SNRPE	-2.7	Small nuclear ribonucleoprotein polypeptide E
SNRPF	-3.0	Small nuclear ribonucleoprotein polypeptide F
SUPV3L1/SUV3	-2.2	RNA, DNA helicase with nuclear and mitochondrial functions
TAL1/SCL	-2.3	T-cell acute lymphocytic leukemia 1 transcriptional regulator

TET3	-1.6	Tet family oncogene -meC demethylase -epigenetic re-programming in mice
TGIF1	-1.7	TGFB-induced factor homeobox 1
TOPBP1	-1.7	Topoisomerase II binding protein 1
TREX1/DNAseIII	-2.1	Three prime repair endonuclease
TRIM28/KAP1	-2.4	Heterochromatin protein, binds HP1
UBA52	-1.6	Ubiquitin-ribosomal L40 fusion protein
XAB2	-3.5	NER, XPA binding protein

The hit-list was then expanded by raising the Z-score cut-off to -1.5. This was done for two reasons: Firstly, as can be seen in Figs 4.1 and 4.2, the data quality for the screens was not perfect and it is likely that possible, novel HR genes may be excluded from the hit list with a more rigorous cut off. This reasoning is strengthened upon examination of Fig 4.4, which shows a number of known HR genes falling above the initial -2 cutoff, most notably RPA1, in both screens. Secondly, the capacity for follow-up of the initial hits was defined by the throughput of the DR-GFP assay. Given the demonstrated capacity for high-throughput, it was of little extra effort to increase the number of preliminary hits for hit confirmation and validation. Also included in the validation list at this point was BRD1 (Z-score -1.3 in 6nM screen), despite being outside the cutoff threshold, I believed it was an interesting but poorly characterised chromatin remodelling protein that could readily be accommodated in the follow up work.

Hit validation

Initial hit validation, to confirm the observations of the initial screens, was performed in the DR-GFP assay at and siRNA concentration of 6nM with a combined list of preliminary hits (all those giving a Z-score of <-1.5 as well as BRD1, Z-score -1.3) taken from both the 10nM and 6nM screens (Fig 4.5 (a)). Most siRNAs selected as preliminary hits from the screens and re-tested gave a decrease in DR-GFP assay activity (those not giving a reduction with a p value of $p < 0.05$ are marked in red) providing confidence in the initial hit screen and hit selection process and although the selected hit c9orf102 (see below) did not produce a statistically significant reduction in the DR-GFP assay, an inhibitory effect was later confirmed in the siRNA deconvolution experiments (Fig 4.7). The ranking of the siRNAs in the DR-GFP validation experiment did not recapitulate that seen in the screen, which may reflect the noise in the initial screening dataset and experimental variability (e.g. variable knockdown efficiency and I-SceI transfection). Further analysis of the validation data was performed by plotting the normalised percentage of GFP positive cells versus normalised cell count data (Fig 4.5 (b)) to determine if there was any correlation between the inhibition of the DR-GFP assay and siRNA-induced toxicity. These data confirmed that not all hits were toxic, though many had an apparent effect on cell viability as might be expected from perturbation of HR. The concern that a large number of toxic siRNAs would be selected in the data analysis as false positives was thus dismissed.

In order to complete the validation of the screen hits, those siRNAs which

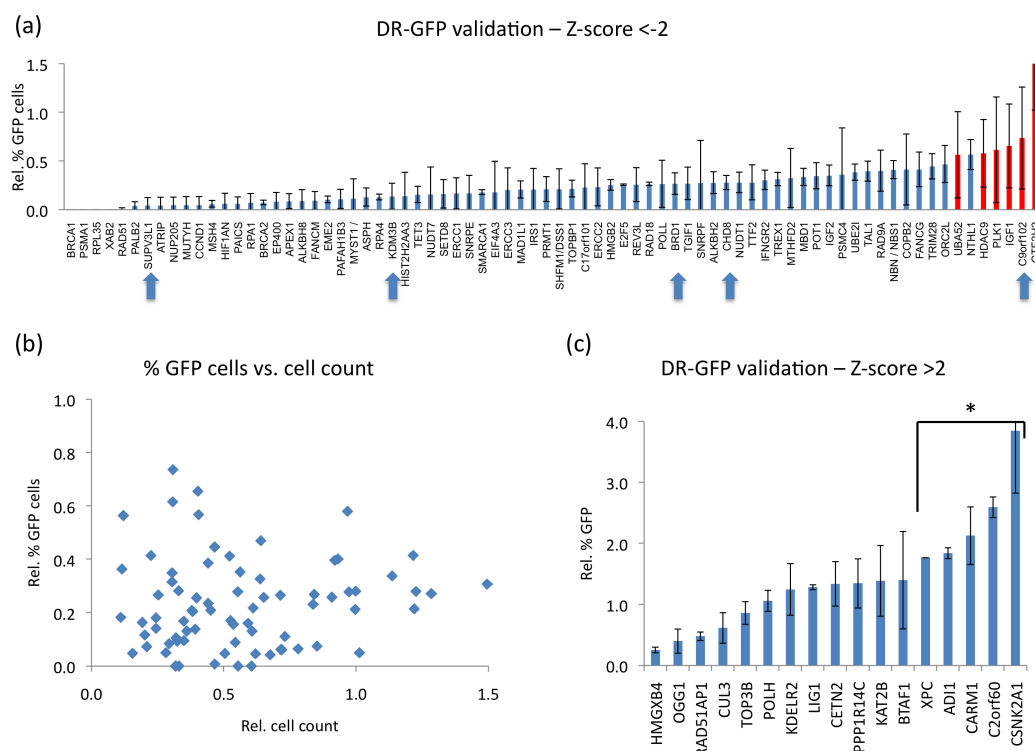


Figure 4.5: siRNA hit validation. **(a)** DR-GFP assay performed with preliminary hits from the siRNA screen in UGFP1 cells. Those with a p value of $p > 0.05$ are highlighted in red. Hits later selected for follow-up are highlighted with blue arrows. **(b)** Plot of relative percentage of GFP positive cells versus relative cell count (obtained during the FACS based screen) for the hit validation experiment. **(c)** Validation of all those siRNAs leading to an increase in HR activity in the original 6nM and 10nM screens in the DR-GFP assay in UGFP1 cells. Those with a p value of $p < 0.05$ are bracketed with a *.

led to an increase (Z-score greater than 2) in the DR-GFP screen were re-tested in the same assay (Fig 4.5 (c)), with only five giving a significant increase in activity ($p > 0.05$). Such siRNAs were not the focus of this particular study and were not pursued further. Far fewer siRNAs were identified at this end of the dataset, suggesting a bias in the assay towards identifying siRNAs with an inhibitory effect on HR or that few of the proteins whose knockdown are targeted by the siRNA library have an inhibitory effect on HR. This skewing of the data may also reflect the composition of the siRNA library used in this study, with a predominance of DNA repair factors.

Further validation of the preliminary hits was conducted at the higher siRNA concentration of 10nM, to maximise protein knockdown and continued by testing for their effect on the cell cycle profile of the cells. The off-target effects of the NT siRNA witnessed in the DR-GFP screen were less of a concern when working on a smaller number of samples as the replicate data quality of these smaller scale experiments was improved. These off-target effects were only observed in the DR-GFP assay and none of the others herein described. It was important to establish that the hits were not exhibiting their inhibitory effect on the DR-GFP assay due to an arrest of the cells in the G1 phase of the cell cycle. Such an effect would render HR impossible and give rise to a false positive in this assay. In general (Fig 4.6 (a)), G1 arrest was not observed for any of the siRNAs, although a number of siRNAs did cause the accumulation of cells in G1 (greater than 70% G1 cells). Only one of those siRNAs generating a G1 phase bias (BRD1) was selected for

further work.

The final stage of the validation process was performed with selected siRNAs of most interest from the preliminary hit list. The selection criteria at this point were for these targets that represented possible novel (i.e. not already linked to repair) HR proteins for which a plausible mechanism could be hypothesised. For example, growth factors that had been identified as preliminary hits were discarded from further analysis. This refined preliminary hit list was then used to perform a DSB inducing agent sensitisation assay (Fig 4.6 (b)). Any siRNAs leading to sensitisation to DSB induction could then be considered to target possible HR proteins. Following a 24 hour reverse transfection with the selected siRNAs, cells were treated with the bifunctional alkylating agent mitomycin C (MMC) at 50ng/ml, which generates inter-strand DNA crosslinks (ICLs) leading to DSB formation in a replication dependent manner. Cells were exposed continually to MMC for 120 hours before determination of cell viability with the resazurin assay (see Materials and Methods). Such ICLs require both HR and ICL repair pathways for repair and inhibition of HR leads to sensitisation of cells to this agent, as may be seen for the RAD51 siRNA treated cells (Fig 4.6 (b)). The normalised effect of each selected siRNA on cell viability is plotted in Fig 4.6 (b). Alongside the RAD51 positive control, a number of siRNAs led to strong sensitising effects and the majority gave some sensitisation to MMC treatment, consistent with the DR-GFP data. The most profound effect was seen with the siRNA targeting the gene for the predicted DNA helicase

c9orf102/RAD26L, see below. These data are suggestive of a role in HR for a number of the siRNAs captured from the screen, however it is noted that such a sensitisation assay, based upon cell viability at a single concentration of MMC may not be effective across all the siRNAs tested. Nevertheless, these data provide a useful, high-throughput assessment of the validity of the siRNA screen.

4.2 siRNA hit deconvolution

Hit selection

Having defined a preliminary hit list from the 10nM and 6nM siRNA screens and with confidence in their ability to inhibit the DR-GFP assay and in many cases, sensitise cells to mitomycin C treatment, it was now necessary to manually curate the list to a more manageable number for hit deconvolution and initial characterisation. The selection criteria were as follows and in line with my research priorities. The project aim was to identify novel HR proteins, so siRNAs targeting genes for proteins which had no known, or a poorly characterised role in HR, were prioritised, with initial emphasis on completely uncharacterised proteins (in particular, c9orf102; see below). Furthermore, it was important to be able to visualise a prospective role within HR for these hits whether direct, or indirect. For example DNA translocase activity may have a direct role in HR (e.g. RAD54) whereas chromatin modification could have direct or indirect roles (e.g. RNF8 mediated H2A ubiquityla-

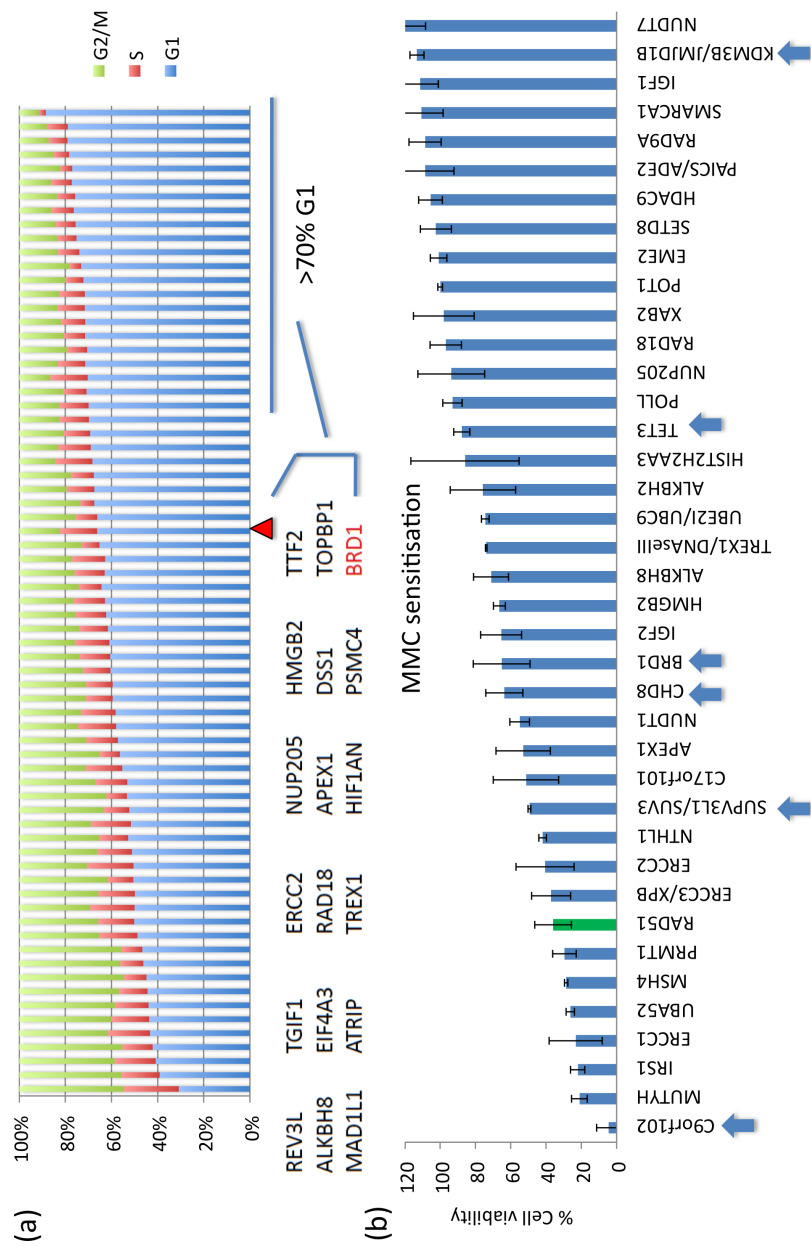


Figure 4.6: Further siRNA hit validation: **(a)** Cell cycle profiles for U2OS cells treated with the preliminary siRNA hits as defined in Fig 4.4 (with the exception of siRNAs targeting known HR genes). The NT siRNA control is marked with a red arrow. **(b)** MMC sensitisation effects of selected siRNAs from the preliminary hit list. U2OS cells were treated with the pooled siRNA for 24 hours before a 120 hour continuous MMC treatment (50ng/ml). Cell viability was measured using the resazurin assay and normalised cell survival values calculated using the NT siRNA treated cells as a control. Hits selected for further follow up are marked with arrows. The RAD51 control siRNA is highlighted in green. Error bars represent the standard deviation of two replicates.

tion or global heterochromatin changes respectively). With these criteria in mind, the hit list for further work was defined as BRD1, c9orf102, CHD8, JMJD1B, TET3 and SUV3. In brief, the known/predicted functions of these selected hits, are annotated in Table 4.2 and constitute (unsurprisingly, given the nature of the siRNA library) a mix of DNA helicases, chromatin remodellers and chromatin binding proteins. The knowledgebase for these hits varies from completely uncharacterised (c9orf102) to well characterised both biochemically and cell biologically (SUV3) [136, 135, 180, 162].

The selected hits were not necessarily the top ranked from the initial screen data analysis. This may be rationalised when one considers that the Z-score ranking takes into account not just the magnitude of the observed effect of each hit, but also the data quality of that particular datapoint, i.e. siRNAs giving a large decrease in HR but for which replicate data quality were poor, will come lower down the hit list than if only the magnitude of their phenotype was taken into account. Furthermore, a number of hits near the top of the initial hit-list were targeting known HR genes and were accordingly of little interest for follow up work. There were a number of known BER and NER proteins amongst the hits but as my focus was on poorly characterised proteins, I elected not to follow these up, though they would serve as interesting targets for further work within our laboratory.

Table 4.2: Selected hits from DR-GFP screen, together with known functions. Predicted protein functions have been assigned according to their annotation in PubMed with specific references, where relevant, included.

ID	target	description
BRD1	predicted bromodomain containing protein	binds acetylated lysine residues as part of HAT complex [138]
c9orf102/ RAD26L	predicted SF2 DNA helicase with homology to RAD26	not characterised
CHD8	chromodomain helicase	transcription and gene expression
JMJD1B	predicted histone lysine demethylase	not characterised
TET3	tet oncogene family member, methyl-cytosine demethylase	epigenetic reprogramming [62]
SUV3	nuclear and mitochondrial DNA and RNA helicase	mitochondrial RNA processing, implicated in DNA repair [136, 135, 180, 162]

4.2.1 siRNA hit deconvolution

With the final hit-list defined, my initial priority was to 'deconvolute' the siRNA reagents that had been used in the screen, namely, siRNA pools of four individual siRNAs all targeting the same transcript. To this end, each of the four siRNAs constituting the original siRNA pool that was used in the initial screen was purchased (Qiagen) and tested individually, alongside the pooled siRNA reagent for their effect on the DR-GFP assay and, importantly, transcript levels by quantitative real-time PCR (qPCR). The deconvolution data are shown in Figs 4.7 and 4.8 and are discussed below. It is common practice in siRNA screens to consider a hit validated if 2 or more of the pooled siRNAs against one target give rise to the same phenotype.

Deconvolution: DR-GFP

With reference to Fig 4.7, all DR-GFP (and most subsequent) deconvolution experiments were conducted with an analogous layout consisting of the positive control (RAD51 in the case of DR-GFP experiments), the NT siRNA and a blank (transfection reagent only) alongside the four individual siRNAs that constitute the pool as well as the pooled reagent itself. It may be observed at this point that the previously observed effect of NT siRNA on the percentage of GFP positive cells was not as pronounced a problem in the deconvolution experiments as in the screen itself, possibly due to the switch from using the robotic handler to performing experiments by hand. Reassuringly, each of the pooled reagents for all selected hits gave a decrease

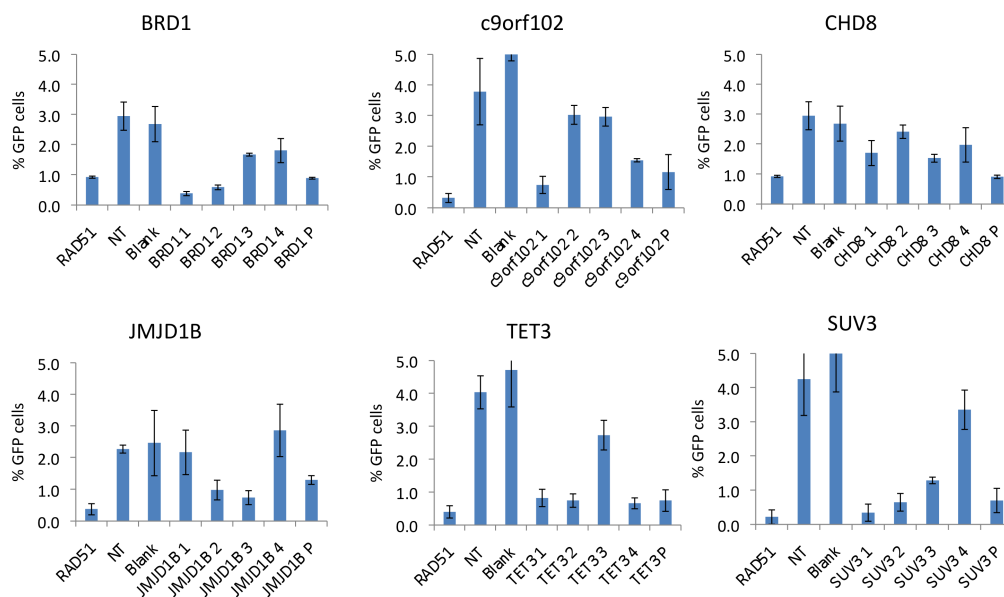


Figure 4.7: Deconvolution of selected hits: DR-GFP assay in UGFP1 cells. The pooled siRNA reagent against each target (the siRNA library consisted of such pools) was deconvoluted into the four individual siRNAs that constitute each pool. Each of these individual siRNAs, along with the pooled reagent, together with NT siRNA and a transfection reagent only control (Blank) were tested alongside the RAD51 positive control. Error bars represent the standard deviation of three replicates.

in the percentage of GFP positive cells in the assay. As had been seen across my studies, variability both within and across experiments remained a problem, though this was countered, to some extent, by a switch of transfection reagents for the I-SceI transfection in later experiments to alleviate problems encountered with the toxicity of PEI. PEI was used in the initial screen due to its low cost. However it has a toxic effect on the cells at the concentrations required for effective transfection. For the lower throughput follow up work, the less toxic and more effective (with regards to induction of GFP in the DR-GFP assay) Lipofectamine 2000 (Invitrogen) transfection reagent was used.

All my selected hits, with the exception of CHD8, produced a marked inhibition of the DR-GFP assay for at least 2 of the individual siRNAs plus the pooled reagent, thus can be considered as validated. Further work was now required to attempt to explain the intra-pool variability, which was addressed subsequently for both JMJD1B and SUV3 (see below). The effect of the CHD8 siRNA was less pronounced than the other hits, with a marginal inhibitory effect from two of the individual siRNAs, probably combining to give the phenotype demonstrated by the pooled reagent.

Deconvolution of selected hits from the siRNA screen: real-time quantitative PCR (qPCR)

In order to define the effectiveness of the siRNA reagents, it was necessary to perform qPCR reactions with each of the siRNAs of each pool to deter-

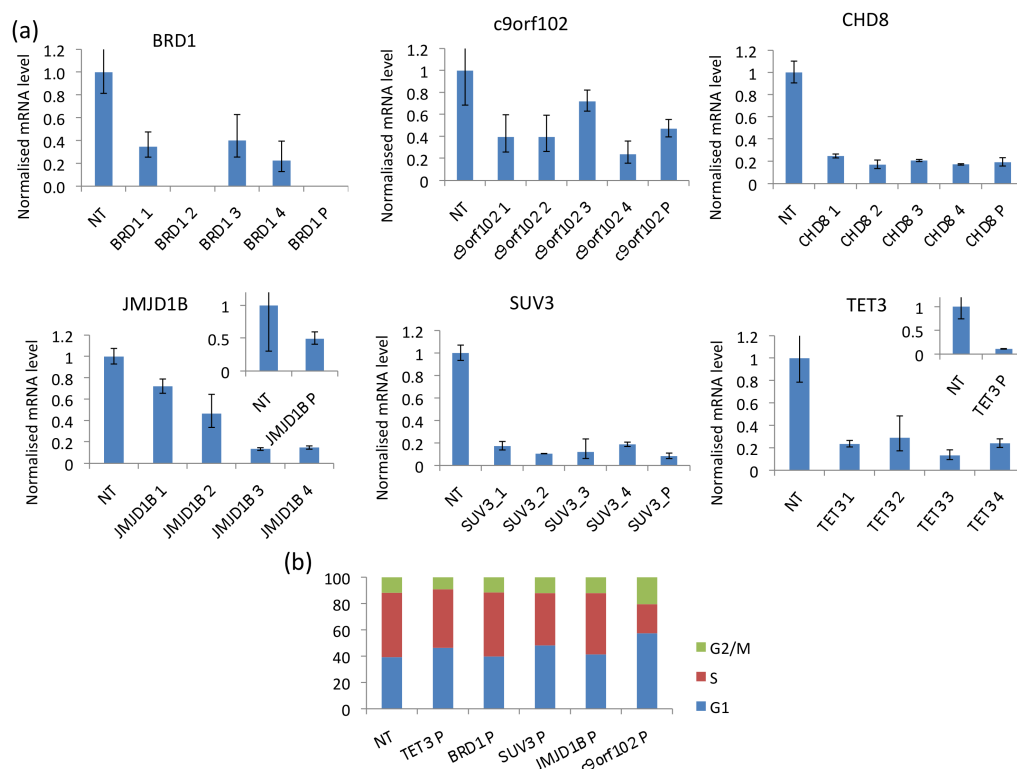


Figure 4.8: **(a)** Deconvolution of selected siRNA hits: real-time qPCR with RNA isolated from U2OS cells. Each of the four constituent siRNAs that make up the pooled siRNA against each target (the pooled reagent was used in the initial screen) were tested alongside the pooled reagent and normalised to their internal GAPDH controls. For each target (BRD1, c9orf102, CHD8, JMJD1B, SUV3 and TET3) the deconvoluted siRNAs and the pool are plotted relative to NT siRNA treated cells. Real-time qPCR was conducted 24 hours following siRNA transfection. Error bars represent the standard deviation of three replicates. **(b)** Cell cycle profiles following 48 hour knockdown of each of the selected hits.

mine if they were knocking down expression levels of the targeted transcript. This was conducted 24 hours after siRNA reverse transfection at a point when target transcript levels should be greatly reduced (target protein levels take longer to show an effect) and with reference to GAPDH as an internal control. All results were normalised to NT siRNA treated cells (Fig4.8). Across all the selected hits, most siRNAs of each pool significantly knocked down target transcript levels. The notable exceptions which gave a limited knockdown were JMJD1B siRNA 1 and c9orf102 siRNA 3 - these particular siRNAs showed limited phenotypes in subsequent assays, which are likely to be explained by their limited effect on transcript levels (see below).

It would be expected that the qPCR data would correlate well with the DR-GFP data and whilst there are those siRNAs that have a limited effect on both target transcript levels and the DR-GFP assay, the correlation is not as strong as could be hoped. The transcript levels were measured at 24 hours after siRNA reverse transfection, though not the same timepoint as the FACS endpoint of the DR-GFP assay (96 hours following siRNA reverse transfection), this timepoint reflects a timepoint at which effective transcript knockdown by RNAi is expected in advance of protein knockdown (protein knockdown previously observed between 48-96 hours after siRNA reverse transfection, see Method Development). Nonetheless, it was encouraging to note that the majority of the siRNAs used were effective with respect to reduction of target transcript levels, increasing confidence in the screening methodology as a whole. If the hit validation criterion used above is extended

to the qPCR data, all the selected hits validate with respect to transcript knockdown.

4.3 Hit characterisation

With all the selected hits now validated, characterisation could now proceed. This was conducted with each siRNA of the pool, as above, or in some cases using solely the validated, pooled siRNA reagents. It was important to establish that the effects of each of the selected hits upon the DR-GFP assay were not due to cell cycle arrest in the G1 phase. Cell cycle profiles were obtained following knockdown of all the selected hits (Fig 4.8 (b)), none of which induced G1 arrest. The c9orf102 pooled siRNA did lead to a greater accumulation in G1 than the other hits, though this could not be considered as G1 arrest and may result from the toxicity of this siRNA pool.

Hits were characterised in a series of different assays. Initially, immunofluorescence data were gathered following target knockdown and a 24 hour camptothecin (CPT) treatment for different molecular readouts (in parenthesis): DNA damage induction and recognition (53BP1), DNA resection (RPA-p34) and nucleofilament formation as a prelude to successful HR (RAD51). Quantification of foci is shown in Fig 4.9 with representative images for the effect of JMJD1B knockdown upon 53BP1-, RPA- and RAD51 foci formation provided in Figs 4.10, 4.11 and 4.12. These data were collected for all the selected hits, but in the first instance (and with reference to Figs 4.7 and 4.8) I will discuss those for BRD1, JMJD1B and TET3, with the remainder

discussed later in this chapter. CHD8 was discarded from further study at this point due to inconclusive validation in the DR-GFP assay. In all cases, 'knockdown' refers to validated knockdown of the transcript of the respective target, and in the case of JMJD1B, its protein product too.

4.3.1 Immunofluorescence

BRD1

BRD1 knockdown leads to a reduction of 53BP1, RPA and RAD51 foci formation with a pattern echoing the DR-GFP deconvolution experiment (Fig 4.9 (a)). It appears from these data that BRD1 siRNAs 1 and 2 and the pool have the most consistent effect in both readouts. The effects on 53BP1 and RPA foci formation are modest, with a more pronounced inhibitory phenotype displayed for RAD51 foci formation.

JMJD1B

Whilst yielding only a modest phenotype in the 53BP1 and RPA foci formation assays (JMJD1B siRNA 2, in particular), JMJD1B knockdown by siRNA 1, 2 and the pooled reagent have a pronounced effect upon RAD51 foci formation, suggesting a genuine negative effect of JMJD1B knockdown upon HR when considered together with the DR-GFP data (Figs 4.7, 4.9 (b), 4.10, 4.11, 4.12). The reduction of RAD51 foci mediated by JMJD1B 1 siRNA is, however, contrary to what would be expected from the real-time qPCR and Western blot data (Figs 4.8 and 4.13), both of which suggest that

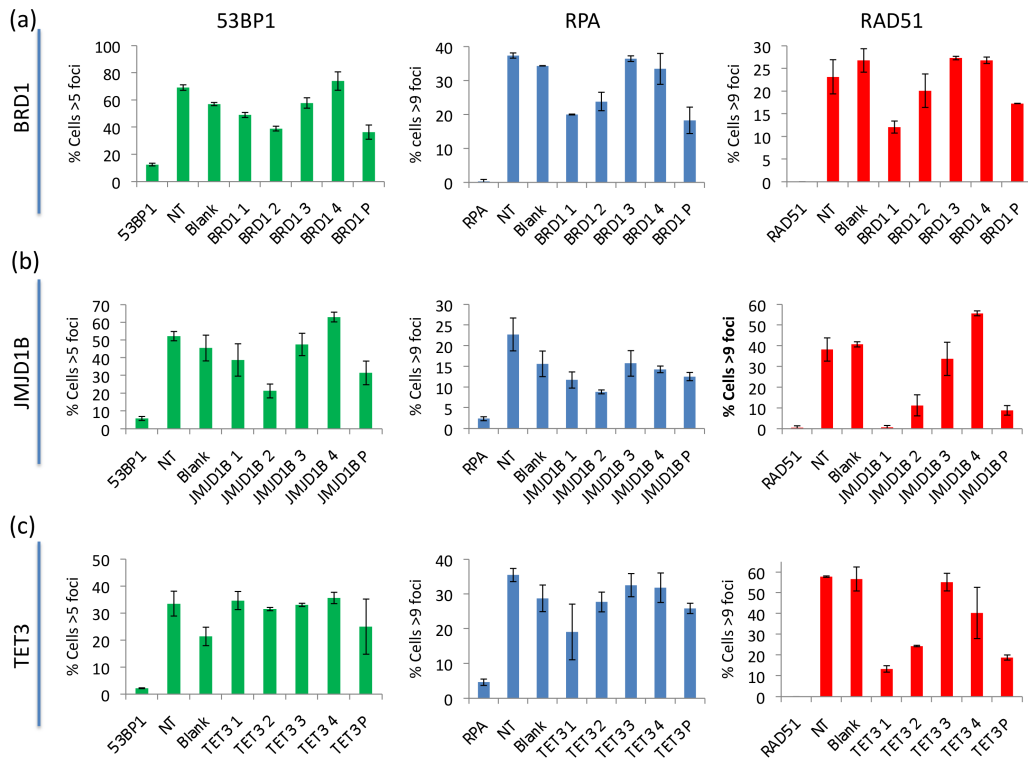


Figure 4.9: Hit characterisation: immunofluorescence in U2OS cells. Effect on 53BP1-, RPA p34 (RPA)- and RAD51- foci following siRNA transfection (48 hours) and induction of DNA damage by 24 hour camptothecin (50nM) treatment. Plots in (a), BRD1, (b), JMJD1B and (c), TET3 show the deconvolution of the four individual siRNAs of each pool, alongside the pooled siRNA, NT siRNA, a transfection reagent only control (Blank) and appropriate positive control. % cells greater than 5- (53BP1) or 9- foci (RPA-p34, RAD51) are plotted, as scored by the image analysis software of the InCell Analyser 1000 (GE). DAPI staining used to identify nucleus, only nuclear foci counted. Error bars represent the standard deviation of two replicates. Each replicate represents the combined data from four images taken from one replicate well of a 96-well plate.

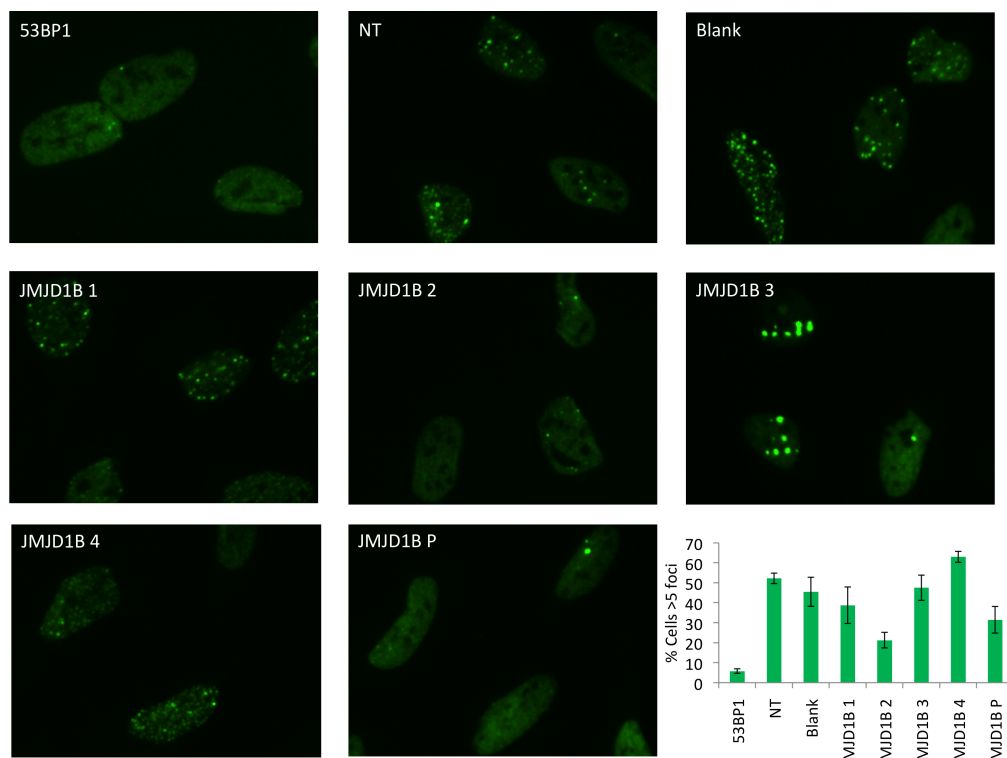


Figure 4.10: Immunofluorescence: JMJD1B siRNA deconvolution showing representative images and quantification of nuclear foci formation in U2OS cells following 48 hour siRNA treatment and 24 hour CPT treatment (50nM). Each siRNA constituting the JMJD1B pool is tested, together with the pooled reagent alongside 53BP1, NT and blank controls. The relevant quantification is shown in the bottom right corner. Pseudocoloured images prepared from the grayscale originals using ImageJ software. Error bars represent the standard deviation of two replicates. Each replicate represents the combined data from four images taken from one replicate well of a 96-well plate.

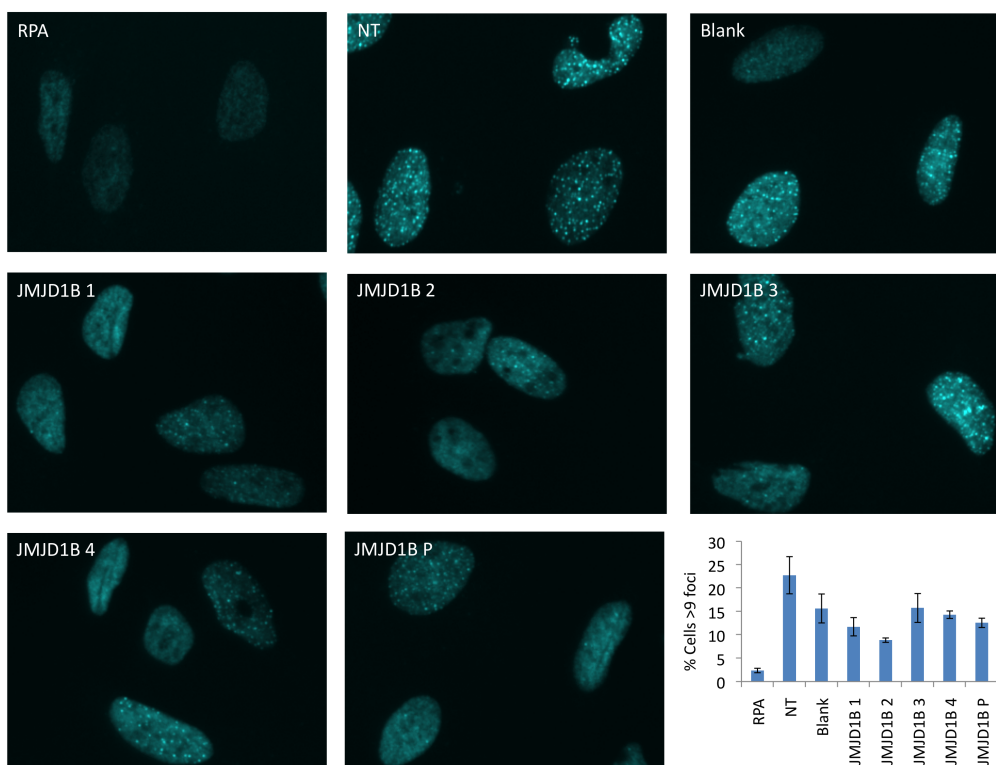


Figure 4.11: Immunofluorescence: JMJD1B siRNA deconvolution showing representative images and quantification of nuclear foci formation in U2OS cells following 48 hour siRNA treatment and 24 hour CPT treatment (50nM). Each siRNA constituting the JMJD1B pool is tested, together with the pooled reagent alongside RPA, NT and blank controls. The relevant quantification is shown in the bottom right corner. Pseudocoloured images prepared from the grayscale originals using ImageJ software. Error bars represent the standard deviation of two replicates. Each replicate represents the combined data from four images taken from one replicate well of a 96-well plate.

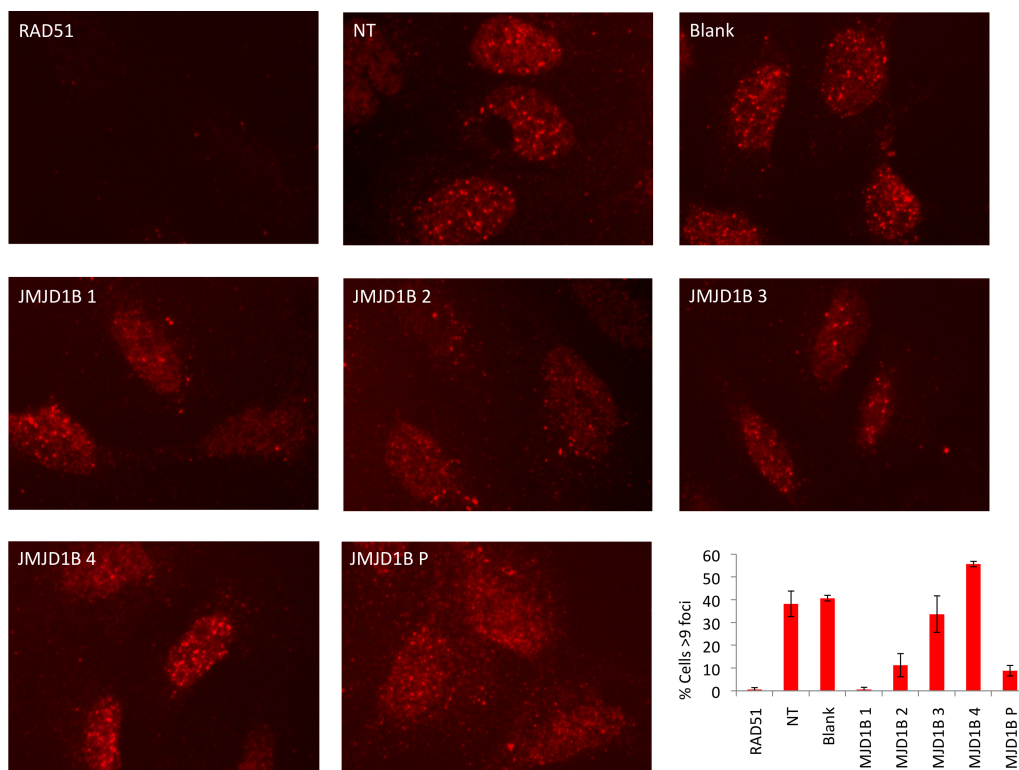


Figure 4.12: Immunofluorescence: JMJD1B siRNA deconvolution showing representative images and quantification of nuclear foci formation in U2OS cells following 48 hour siRNA treatment and 24 hour CPT treatment (50nM). Each siRNA constituting the JMJD1B pool is tested, together with the pooled reagent alongside RAD51, NT and blank controls. The relevant quantification is shown in the bottom right corner. Pseudocoloured images prepared from the grayscale originals using ImageJ software. Error bars represent the standard deviation of two replicates. Each replicate represents the combined data from four images taken from one replicate well of a 96-well plate.

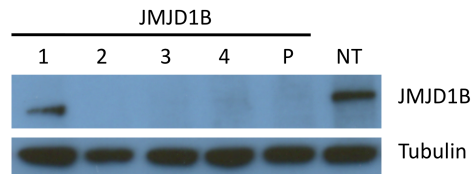


Figure 4.13: JMJD1B Western blot with cell extracts taken from U2OS cells treated for 48 hours with all four JMJD1B siRNAs and the pooled reagent, alongside a NT siRNA control.

all the siRNAs except JMJD1B 1 knockdown JMJD1B protein levels.

TET3

TET3 knockdown has little effect on 53BP1 and RPA foci formation, but leads to a significant reduction in RAD51 foci formation, in particular for TET3 siRNAs 1, 2 and the pool (Fig 4.9 (c)). The pattern of RAD51 foci data echoes that seen in the DR-GFP assay (Fig 4.7), lending support to a possible role of TET3 in HR.

Taken together with the DR-GFP and qPCR deconvolution data, the immunofluorescence experiments serve to both highlight those siRNAs that have a robust effect on HR across multiple assays, increasing confidence in the assumption that the targets of these siRNAs are play a role in HR, whilst illuminating some inconsistencies across the different assays. The assay to assay variability is discussed in further detail in Chapter 6.

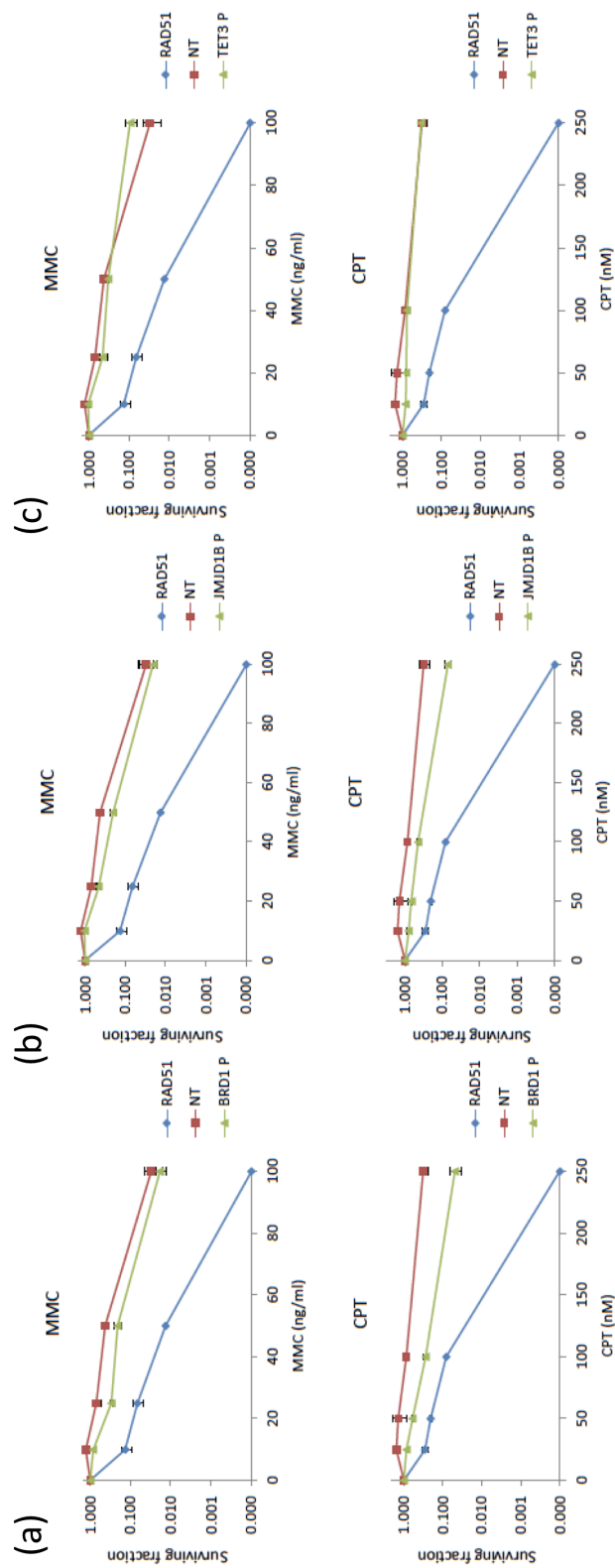


Figure 4.14: siRNA hit characterisation: clonogenic survival assays in U2OS cells. Clonogenic survival curves for U2OS cells treated with titrations of mitomycin C (MMC) or camptothecin (CPT). Cells were reverse transfected with either (a) BRD1, (b) JMJD1B or (c) TET3 pooled siRNAs as well as NT and RAD51 siRNA controls before a 24 hour compound treatment. Colonies were left to form over ten days and those with >50 cells were then scored manually. Surviving fractions were calculated relative to the relevant untreated control. Error bars represent the standard deviation of two replicates.

4.3.2 Clonogenic survival

To further underscore possible functions for BRD1, JMJD1B and TET3 in promoting/enabling HR, I wanted to determine if knockdown of any of these targets rendered cells sensitive to DNA damaging agents as assayed by clonogenic survival. Cells that had been transfected with pooled siRNA against a given target were plated and exposed to a 24 hour DNA damage-inducing drug treatment, washed to remove genotoxin and then left for colonies to form. The DNA damage inducing drugs chosen both lead to replication-dependent DSB formation (a major pathway for the generation of HR substrates) but via distinct mechanisms. Mitomycin C (MMC) generates inter-strand DNA crosslinks [89] whilst camptothecin (CPT) is a topoisomerase I inhibitor that traps topoisomerase I in its DNA bound ternary complex, preventing ligation of the topoisomerase induced DNA break and allowing replication dependent DSB formation [78]. See Discussion Chapter for discussion on the choice of DNA damaging agents.

Fig 4.14 demonstrates the sensitising effect of BRD1, JMJD1B and TET3 siRNA transfected cells towards both MMC and CPT. Whilst TET3 knockdown confers no sensitising effects, BRD1 and JMJD1B knockdown sensitises cells to both MMC and CPT treatments in the colony formation assays. The degree of the sensitising effect in each case was significant, but not dramatic and both BRD1 and JMJD1B knockdown appear to sensitise U2OS cells to a greater extent with respect to CPT treatment as opposed to MMC. These sensitising effects for BRD1 and JMJD1B siRNA knockdowns, in combina-

tion with the DR-GFP and RAD51 foci formation data shown above (Figs 4.7 and 4.9) are all consistent with both BRD1 and JMJD1B being required for efficient HR. Given the previously favourable data collected for TET3 in the DR-GFP and foci formation assays, it is surprising that a sensitisation to MMC or CPT is not seen.

4.4 c9orf102

4.4.1 Initial characterisation

One of the hits that was of particular interest was the uncharacterised protein c9orf102 or RAD26L. Initial characterisation of c9orf102 was conducted based upon its predicted sequence (Fig 4.15) and reveals some similarity to *S.cerevisiae* Rad26 (28% amino acid sequence identity). *S.cerevisiae* Rad26 is a DNA helicase involved in transcription coupled nucleotide excision repair and shows functional and sequence similarity to its human homologue ERCC6 (or Cockayne Syndrome B, CSB) [206]. However, given the limited sequence identity, it would be hasty to draw a direct functional similarity between *S.cerevisiae* Rad26 and c9orf102. What is perhaps more certain is that c9orf102 may be characterised as a potential helicase/translocase due to the presence of conserved SNF2, DEAD box and helicase domains 4.15. The c9orf102/RAD26L predicted protein is also highly conserved in higher mammals, from *Mus musculus*, *Rattus norvegicus* and through to *Pan troglodytes* and *Homo sapiens* (BLAST search and sequence alignment using Geneious

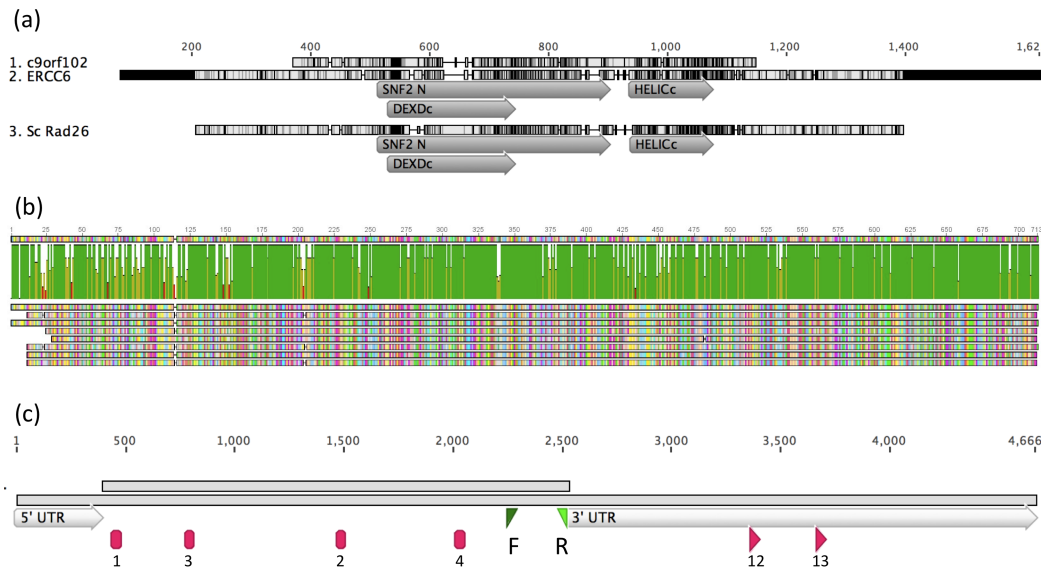


Figure 4.15: Sequence analysis of c9orf102 performed using Geneious software: **(a)** Protein sequence alignment of c9orf102 with ERCC6 and *S.cerevisiae* Rad26. Identical residues in aligned regions are represented by black lines, similar residues in grey. Conserved domains are annotated in grey, SNF2 N = N-terminal SNF2 helicase domain, DEXDc = DEAD box helicase domain, HELICc = helicase c-terminal domain. **(b)** Protein alignment of predicted RAD26L proteins in higher mammals; (from top to bottom) *Homo sapiens*, *Rattus norvegicus*, *Pan troglodytes*, *Canis familiaris*, *Oryctolagus cuniculus*, *Mus musculus*, *Pongo abelli*, *Callithrix jacchus*. Sequence identity is represented as green bars in the scale at the top, with sequence similarity in yellow. **(c)** Schematic of (*TOP*) the c9orf102 coding sequence and (*BOTTOM*) the c9orf102 mRNA with the 5'- and 3'-UTRs annotated. The red blocks and arrows represent the siRNAs against c9orf102 and the green annotations, the primers used for qPCR (F = forward primer, R = reverse primer).

software), suggesting a conserved role for this protein in mammals.

Following successful validation of the c9orf102 siRNA in the DR-GFP and qPCR assays (Figs 4.7 and 4.8), deconvolution of the four individual siRNAs that constitute the c9orf102 siRNA pool was continued in immunofluorescence assays (Fig 4.16 (a)) and demonstrated that knockdown of c9orf102 has a significant effect on both RPA and RAD51 foci formation without affecting 53BP1 foci (foci scored following 48 hour siRNA treatment and subsequent 24 hour CPT treatment). To gain a more detailed view of the kinetics of RAD51 foci formation following c9orf102 knockdown, I also looked at the levels of foci at numerous timepoints following DNA damage induction. c9orf102 siRNAs 1 and 4 as well as the pooled reagent caused a significant and lasting reduction in RAD51 foci levels at all timepoints (Fig 4.16 (b)). Taken as a whole, the foci data for c9orf102 deconvolution demonstrate that c9orf102 siRNAs 1, 4 and the pooled reagent have an effect across the RPA and RAD51 foci formation assays and RAD51 foci timepoint experiment. The variable results for siRNAs 2 and 3 across the immunofluorescence experiments suggest that these two siRNAs do not have a strong, consistent effect on HR - this is in line with the data seen in the DR-GFP and qPCR assays (Figs 4.7 and 4.8).

Analysis of the c9orf102 deconvolution experiments show that siRNAs 1, 4 and the pool all gave clear effects consistent with HR inhibition (Figs 4.7 and 4.16). c9orf102 siRNA 1 and the pool were seen in these assays to be noticeably toxic, whereas siRNA 4 was not. For this reason c9orf102 siRNA

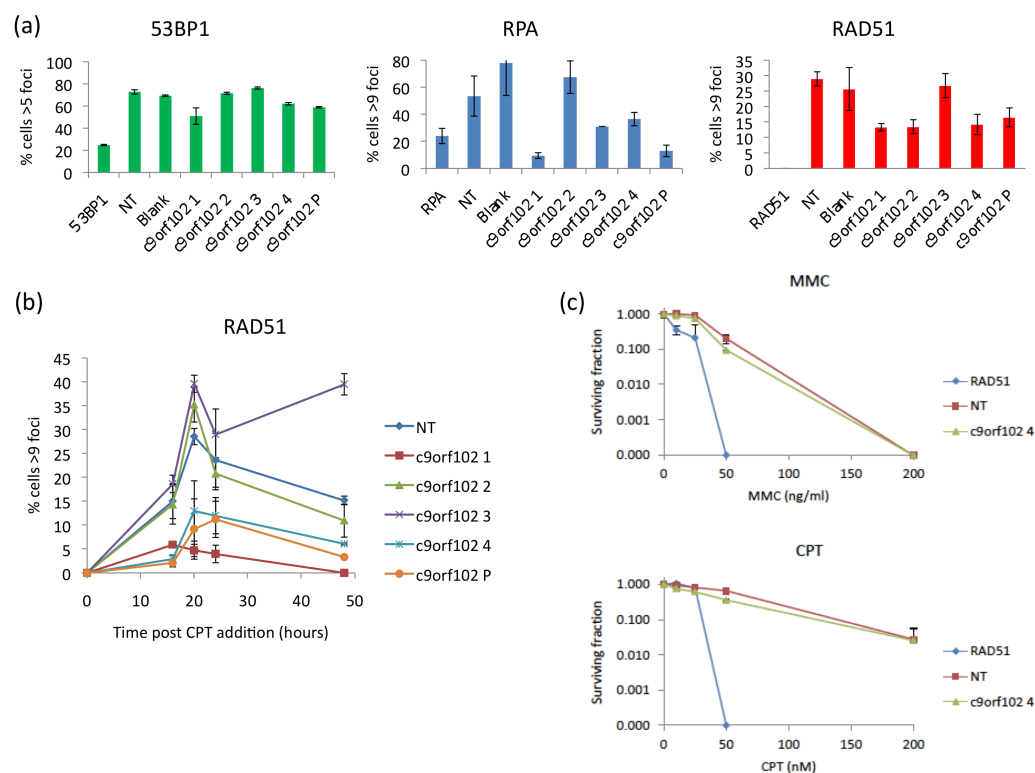


Figure 4.16: c9orf102 characterisation. **(a)** Deconvolution of c9orf102 siRNA in 53BP1-, RPA- and RAD51-foci formation assays in U2OS cells and, **(b)** RAD51 foci formation timepoint assay. **(c)** Clonogenic survival of c9orf102 4 siRNA transfected U2OS cells treated with MMC and CPT. Error bars represent the standard deviation of two replicates.

4 was selected for clonogenic survival assays to assess the sensitising effect of c9orf102 knockdown to the DNA damaging agents MMC and CPT (Fig 4.16 (c)). These data show a very slight sensitisation to both MMC and CPT upon c9orf102 knockdown, consistent with previous data, although a more striking phenotype in these survival assays was expected.

4.4.2 Rescue experiments.

In order to demonstrate that the phenotype recorded for any particular siRNA in an assay is not mediated by an off-target effect, it is necessary to rescue the phenotype by exogenous expression of the wild-type protein in cells that have had the endogenous protein knocked down. To facilitate such a series of rescue experiments, I needed either a siRNA-resistant c9orf102 expression vector, or siRNAs that target the 3' untranslated region (3'-UTR) of the c9orf102 transcript. Fortunately, the latter is available due to the presence of an extensive 3'-UTR in the predicted c9orf102 transcript (Fig 4.15). Two such 'rescue' siRNAs were obtained (c9orf102 12, c9orf102 13) and tested for their effects in the DR-GFP, qPCR, RAD51 foci and DNA damage sensitisation assays (Fig 4.17). These data suggest that c9orf102 13 siRNA effectively knocks down c9orf102 levels leading to inhibition of the DR-GFP assay and greatly reduced RAD51 foci formation following DNA damage - consistent with the observed phenotype of c9orf102 knockdown using other siRNAs. Importantly, c9orf102 13 did not exhibit the toxic effects exerted by c9orf102 1 and the pooled reagent (whose toxicity is likely to

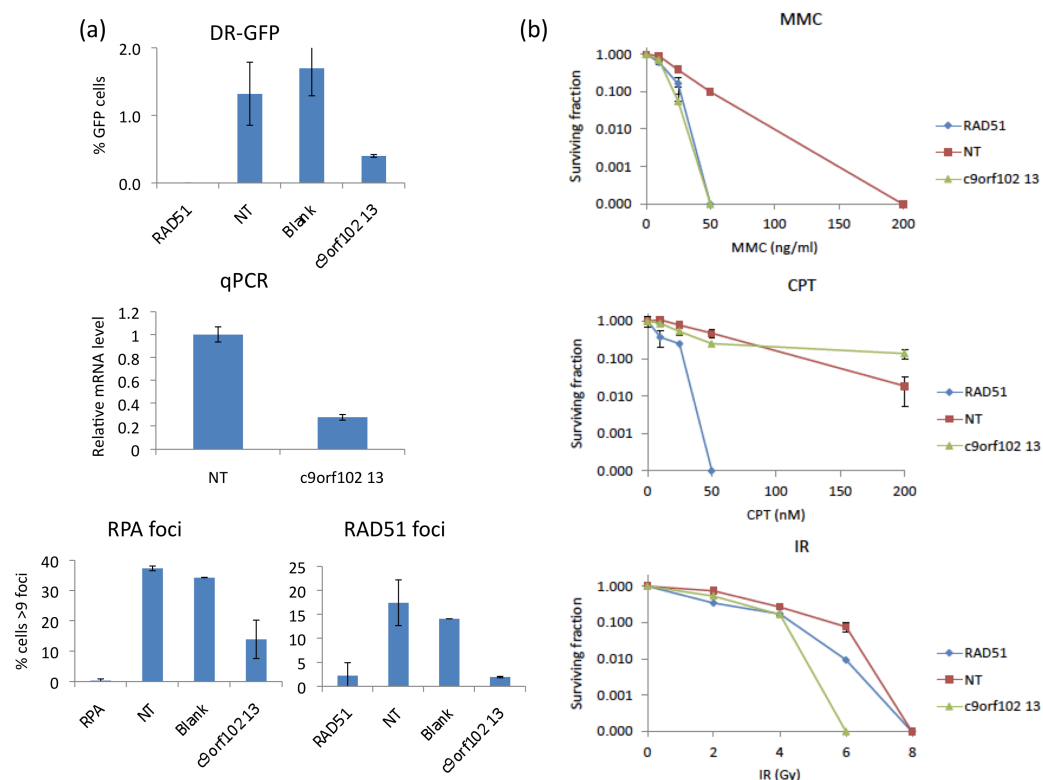


Figure 4.17: c9orf102 rescue siRNA characterisation. **(a)** Testing of the c9orf102 13 rescue siRNA that targets the 3'-UTR of the predicted c9orf102 transcript in the DR-GFP (UGFP1 cells), real-time qPCR (qPCR) and both RPA and RAD51 foci formation assays (U2OS cells). **(c)** Clonogenic survival of c9orf102 13 siRNA transfected U2OS cells treated with MMC, CPT and γ irradiation. Error bars represent the standard deviation of two replicates.

be driven by c9orf102 1). Accordingly, clonogenic survival assays were conducted following c9orf102 knockdown by c9orf102 13 (Fig 4.17 (b)). These assays show sensitisation of c9orf102 deficient cells to MMC and (to a lesser extent) CPT as well as to treatment with γ irradiation. Taken together, the data for siRNA c9orf102 13 continue to support a role for c9orf102 in HR.

Using the c9orf102 13 rescue siRNA, I conducted a series of experiments to determine whether a His-tagged c9orf102 expression vector, His-c9orf102 (Qiagen) was capable of rescuing the HR-deficient phenotype demonstrated by treatment of cells with c9orf102 13 siRNA (Fig 4.18). It was apparent from the initial DR-GFP rescue experiment (Fig 4.18 (a)) that there was no rescue of the decrease in GFP positive cells caused by c9orf102 knockdown, a possible explanation for this could be the protocol used in which the His-c9orf102 vector was transfected into cells 24 hours prior to I-SceI transfection. With such a protocol, it is not possible to ensure that all cells have been transfected with both the rescue vector and I-SceI. To try to counter this technical problem, I repeated the experiment by co-transfecting the I-SceI together with the rescue vector (Fig 4.18 (b)). This protocol should maximise the number of cells transfected with both vectors but still did not demonstrate the anticipated rescue of the c9orf102 knockdown cells. Despite the lack of a rescue for the phenotype displayed in the DR-GFP assay, I conducted a rescue experiment in the RAD51 foci formation assay (Fig 4.18 (c)) which, in line with the DR-GFP data, showed no rescue in the presence of the His-c9orf102 vector. Interestingly, there was a notable increase in RAD51

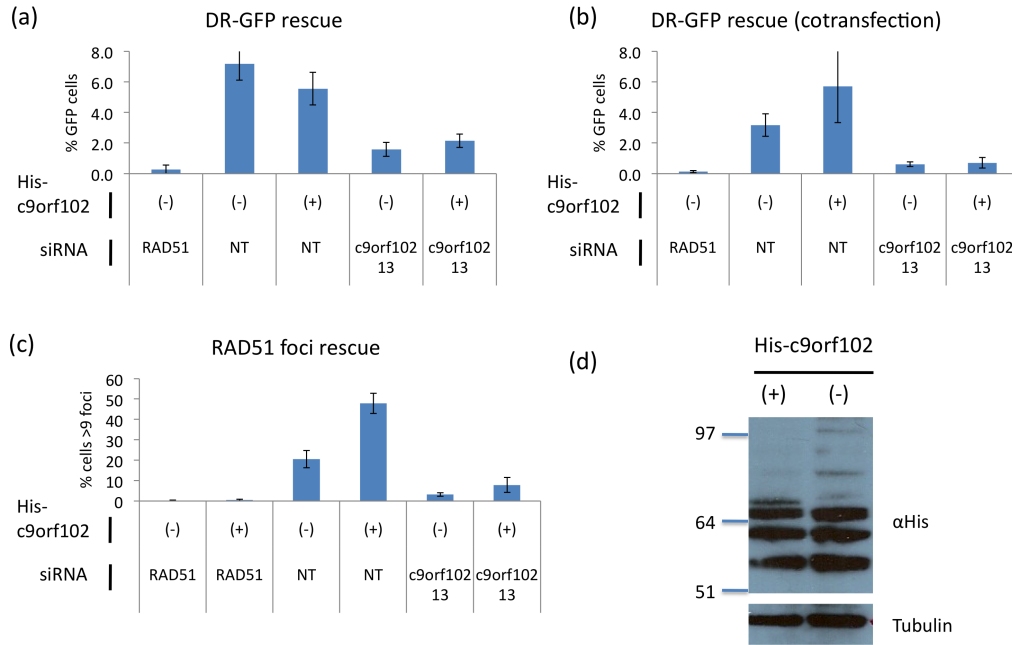


Figure 4.18: c9orf102 rescue experiments: **(a)** DR-GFP rescue experiment in UGFP1 cells with 24 hour siRNA transfection followed by His-c9orf102 transfection (+) or transfection reagent only control (-) at 24 hours prior to I-SceI transfection. **(b)** DR-GFP rescue experiment with 48 hour siRNA transfection before His-c9orf102 (+) or transfection reagent only control (-) transfected at the same time as I-SceI. **(c)** RAD51 foci formation rescue experiment with siRNA transfection of U2OS cells for 24 hours, His-c9orf102 (+) or transfection reagent only control (-) for 24 hours and subsequent CPT (50nM) treatment for 24 hours. **(d)** Western blot with anti His tag antibody in His-transfected (+) and lipofectamine 2000 only control (-) U2OS cells. c9orf102 predicted protein molecular weight = 81kDa.

foci formation levels in the NT siRNA treated cells in the presence of the rescue vector - the reason for this is unclear and due to the uncertainties relating to the c9orf102 sequence (see below) has yet to be investigated.

In light of the inability to rescue the HR deficient phenotype in c9orf102 knockdown cells in both the DR-GFP and RAD51 foci formation assays, I tested for the expression of His-c9orf102 transfected cells by Western blot with an anti-His antibody (Fig 4.18 (d)), as may be expected with an antibody against an epitope which may have many endogenous homologues, the anti-His antibody detects multiple bands even in cells not transfected with the rescue vector (Fig 4.18). There was, however, a single band specifically detected in the rescue vector transfected cells with a molecular weight of around 70kDa, this appears to be too small relative to the predicted c9orf102 molecular weight of 81kDa.

At this point, it was important to determine if the siRNAs I had been using were producing knockdown of c9orf102 at the protein level. To address this, Western blots were performed with two commercially available c9orf102 antibodies (Fig 4.19). In neither case was there clear knockdown of any of the bands recognised by each antibody. The number of nonspecific bands in each case suggests the antibodies may not have the requisite specificity to detect c9orf102 effectively.

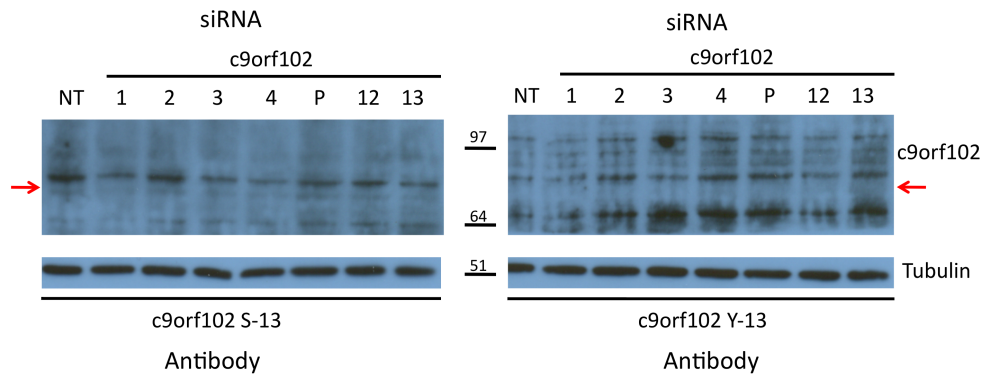


Figure 4.19: c9orf102 Western blot from U2OS cells lysed 48 hours after reverse transfection with the indicated siRNAs. Two different antibodies, c9orf102 S-13 and c9orf102 Y-13 were used, as indicated. Approximate predicted c9orf102 position (81kDa) marked with red arrows.

4.4.3 c9orf102 Northern blot

The NCBI database predicts a transcript (including 5'- and 3'-UTRs of around 4600 bases. To verify this prediction, I performed a Northern blot (with the assistance of Dr C. Bachrati, Helleday group) using the predicted full length c9orf102 coding sequence as a probe (Fig 4.20). Along with assessing the effect of exposure to DNA damaging agents (MMC, CPT) and cell cycle synchronisation in S-phase (via mimosine induced G1 arrest and subsequent 6 hour release) on c9orf102 transcript expression levels, we determined the length of the c9orf102 transcript and verified its knockdown by c9orf102 13 siRNA. Of immediate note was the length of the transcript, which, at around 7500-8000 bases, far exceeded the predicted length. This result throws into question exactly what sequence is expressed in cells and

whether the predicted mRNA sequence is correct. This result also provides a possible explanation as to why the rescue experiments were unsuccessful and why the Western blot from the extracts of His-tagged c9orf102 expressing cells does not provide convincing results. Investigation of the true nature of c9orf102 is the subject of continuing work in the lab.

4.5 SUV3

My initial siRNA hit deconvolution experiments (Figs 4.7 and 4.8) suggested that knockdown of the mitochondrial and nuclear DNA and RNA helicase SUV3, which has recently been shown to interact with RPA [180, 210], leads to a significant inhibition of HR in the DR-GFP assay. These observations are consistent with what is already known about this multi-faceted helicase and warranted further investigation. Accordingly, I deconvoluted the four SUV3 siRNAs from the pooled siRNA reagent in 53BP1-, RPA- and RAD51- foci formation assays (Fig 4.21 (*TOP*)). These data suggest that whilst not affecting 53BP1 foci formation, SUV3 knockdown leads to significant decreases in both RPA and especially RAD51 foci formation following DNA damage induction. Furthermore, clonogenic survival assays (Fig 4.21 (*BOTTOM*)) showed a marked sensitisation of SUV3 knockdown cells to the DNA damaging agents MMC, CPT and γ -irradiation.

To further validate SUV3 as a potential HR protein I used a verified antibody to SUV3, kindly provided by Prof. Piotr Stepień (University of Warsaw, Poland), allowing Western blot confirmation of SUV3 knockdown

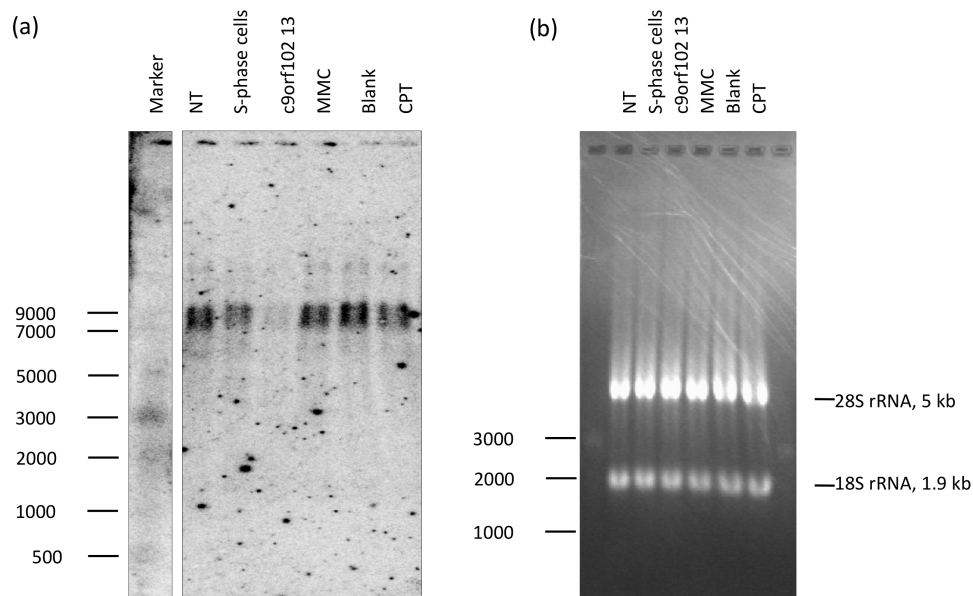


Figure 4.20: **(a)** c9orf102 Northern blot probed with the full length radiolabelled c9orf102-EST and visualised with a Bio-Rad phosphoimager (see Materials and Methods). RNA extracts were taken from NT siRNA transfected, S-phase synchronised (24 hour mimosine arrest followed by 6 hour release), c9orf102 13 siRNA transfected, MMC treated (50ng/ml), untreated and CPT treated (50nM) U2OS cells. Loading control **(b)** showing ribosomal RNA bands on the RNA gel, stained with ethidium bromide.

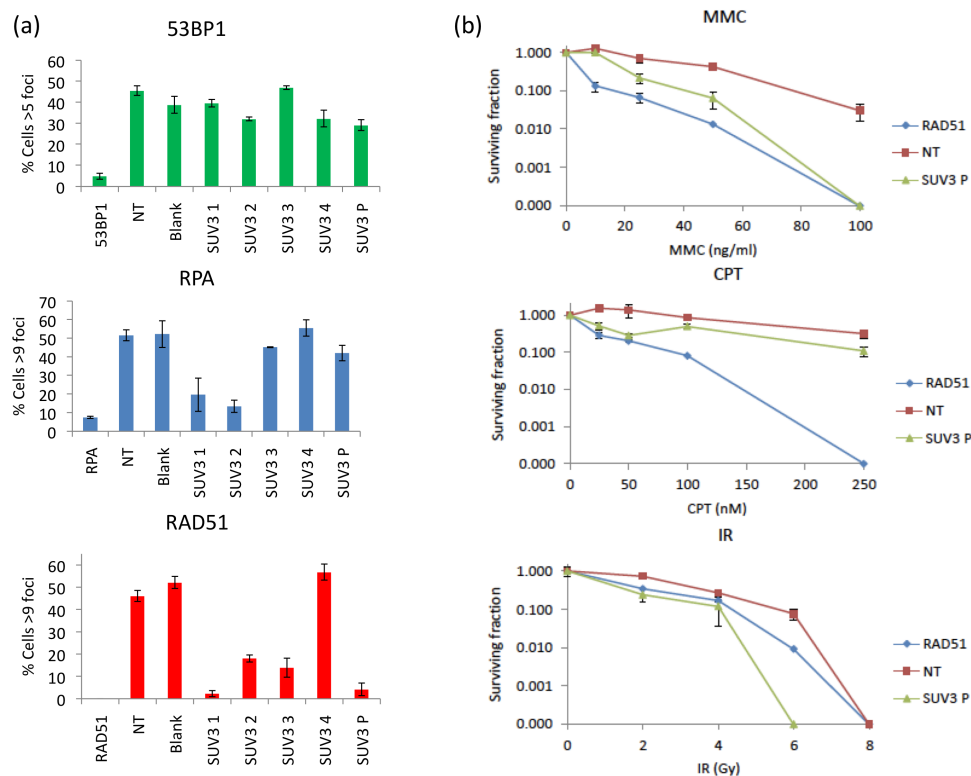


Figure 4.21: Foci formation and colony formation assays following SUV3 knockdown. **(a)** 53BP1-, RPA- and RAD51 foci formation assays following siRNA transfection (48 hours) and 24 hour CPT (50nM) treatment. The following conditions were used for each foci assay - the four individual siRNAs of the SUV3 pool, alongside the pooled reagent, NT siRNA, transfection reagent only (Blank) and the relevant positive controls for each readout and **(b)** clonogenic colony formation assays for SUV3 siRNA (pooled reagent) transfected U2OS cells subsequently exposed to DNA damage by MMC, CPT and γ -irradiation, alongside NT and RAD51 siRNA transfected controls.

by my siRNAs (Fig. 4.22). Along with confirmation of effective SUV3 knockdown by all SUV3 siRNAs used (at least in the case of the 48 hour blot), it was of great interest to note the effect of SUV3 knockdown upon RAD51 protein levels. Throughout my work, I have habitually used RAD51 knockdown as a positive control when performing blots with untested antibodies, in this case it gave a fortuitous insight into how SUV3 knockdown might mediated HR inhibition. The almost complete abolition of RAD51 protein 48 hours after SUV3 siRNA transfection provides just such an explanation for the phenotype demonstrated by SUV3 knockdown in the DR-GFP, RAD51 foci formation and clonogenic survival assays, though not explaining the effect seen on RPA foci formation - whether the recent identification of an RPA:SUV3 interaction [210] sheds any light on this observation is not yet clear. Furthermore, the recovery of SUV3 and RAD51 expression levels in SUV3 4 siRNA-treated cells 72 hours after siRNA transfection may well explain the lack of a phenotype demonstrated for this siRNA in the DR-GFP and foci formation assays.

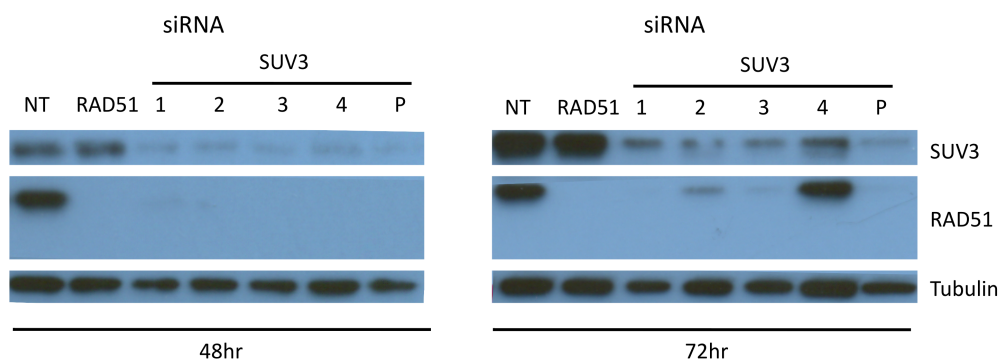


Figure 4.22: SUV3 and RAD51 Western blot at both 48- and 72-hours following siRNA reverse transfection with all SUV3 siRNAs plus RAD51 and NT siRNA controls.

Chapter 5

Results: small molecule screens

Two small molecule screens were conducted and are considered separately in this chapter. Both used the same assay format as detailed in the Materials and Methods and Method Development chapters. Briefly, U2OS cells were seeded at 12,000 cells per well in 96-well plates and left to adhere and grow for 24 hours before treatment of cells with the compound libraries and immediate addition of the I-SceI and transfection reagent mixture. The following day, the media on the cells was changed and the cells left for a further 24 hours at which point they were trypsinised and fixed in 4% PFA for subsequent FACS analysis.

The first screen was conducted using a kinase inhibitor library of 217 compounds (see below for further details). Due to the small size of the library, all steps of this screen were conducted by hand. The second screen used the NCI diversity set I and NCI diversity set II libraries from the National Cancer Institute, numbering 3440 compounds. The larger size of this library

necessitated the use of robotics for the compound treatment steps of the assay, whilst the I-SceI transfection and sample preparation for FACS were carried out by hand. Both screens used compounds at a concentration of $5\mu\text{M}$ together with DMSO vehicle (0.75% v/v) and positive (PCI at 0.4 and $0.6\mu\text{M}$) controls.

Both screens were carried out in triplicate with the average, DMSO normalised % GFP cells calculated for each compound treatment. These data were then used to calculate a Z-score for each compound treatment (see Fig 2.4). The Z-score represents the distance of each datapoint from the mean of the dataset in terms of standard deviations. Each integer Z-score value represents 1 standard deviation away from the mean. The cutoff used to identify a small molecule hit in both the small molecule screens was a Z-score of ≤ -2 i.e. two standard deviations or more below the mean.

Along with the % GFP cell data obtained using the FACS assay, I used the total number of gated, living cells (those with favourable forward- and side-scatter properties), herein described as 'cell number', to give an indication of the toxicity of compounds. Comparison of the cell count data for each compound relative to DMSO control treated cells allowed calculation of a relative cell count as an indication of compound toxicity.

5.1 Kinase inhibitor screen in the DR-GFP assay

The kinase inhibitor library

The first small molecule screen applied a library of kinase inhibitors kindly provided by Professor Stefan Knapp of the Structural Genomics Consortium (SGC), University of Oxford. This small library of 217 compounds represented a targeted screening approach against known druggable targets with, in many cases, well characterised compounds. The advantage of such an approach is that it may yield hits for which the molecular target is known, greatly aiding the understanding of the inhibitory mechanism and further development of any hit compounds. Due to the small size of the library, the screen was conducted by hand and the results are discussed below.

5.1.1 Kinase inhibitor screen

The screen was run with compounds at $5\mu\text{M}$ and analysis of the dataset (Fig 5.1 (a)) shows that the PCI control was effective with respect to inhibition of the DR-GFP assay at all concentrations used and that DMSO at a final concentration of 0.75% (v/v) had a negligible effect upon the percentage of GFP positive cells as compared to blank (untreated) cells. Unfortunately, the data quality of the screen was poor with excessively large differences between replicates, in particular for those compounds leading to an increase in the percentage of GFP positive cells (Fig 5.1 (b)). This is a comparable effect,

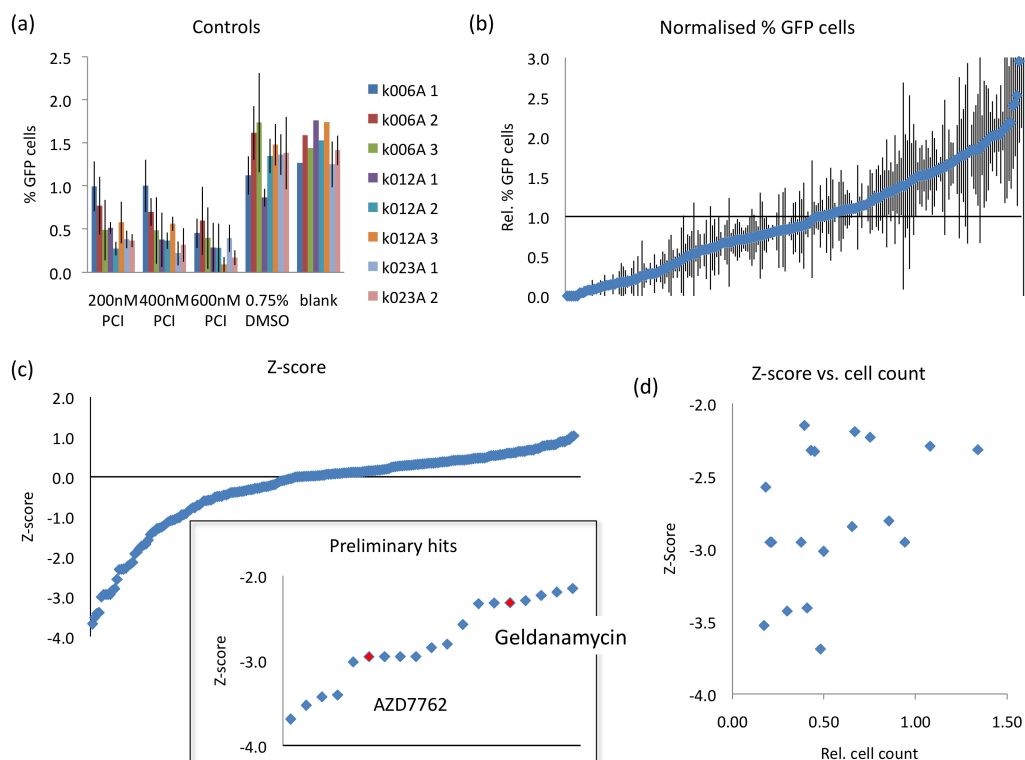


Figure 5.1: Kinase inhibitor DR-GFP screen in UGFP1 cells. **(a)** Control data across all plates for the kinase inhibitor screen conducted in UGFP1 cells with PCI used as a positive control. **(b)** Mean relative % GFP cells for all compounds of the kinase library, normalised to the DMSO control. The x-axis is set at a value of 1.0. **(c)** Calculated Z-scores for the kinase inhibitor library, with the preliminary hits ($Z\text{-score} \leq -2$) shown inset, the HR inhibitor AZD7762 and the HSP90 inhibitor Geldanamycin are marked in red. **(d)** Correlation plot of Z-score values versus relative (DMSO normalised) cell count data collected during FACS. Error bars in (a) and (b) represent the standard deviation of three replicates.

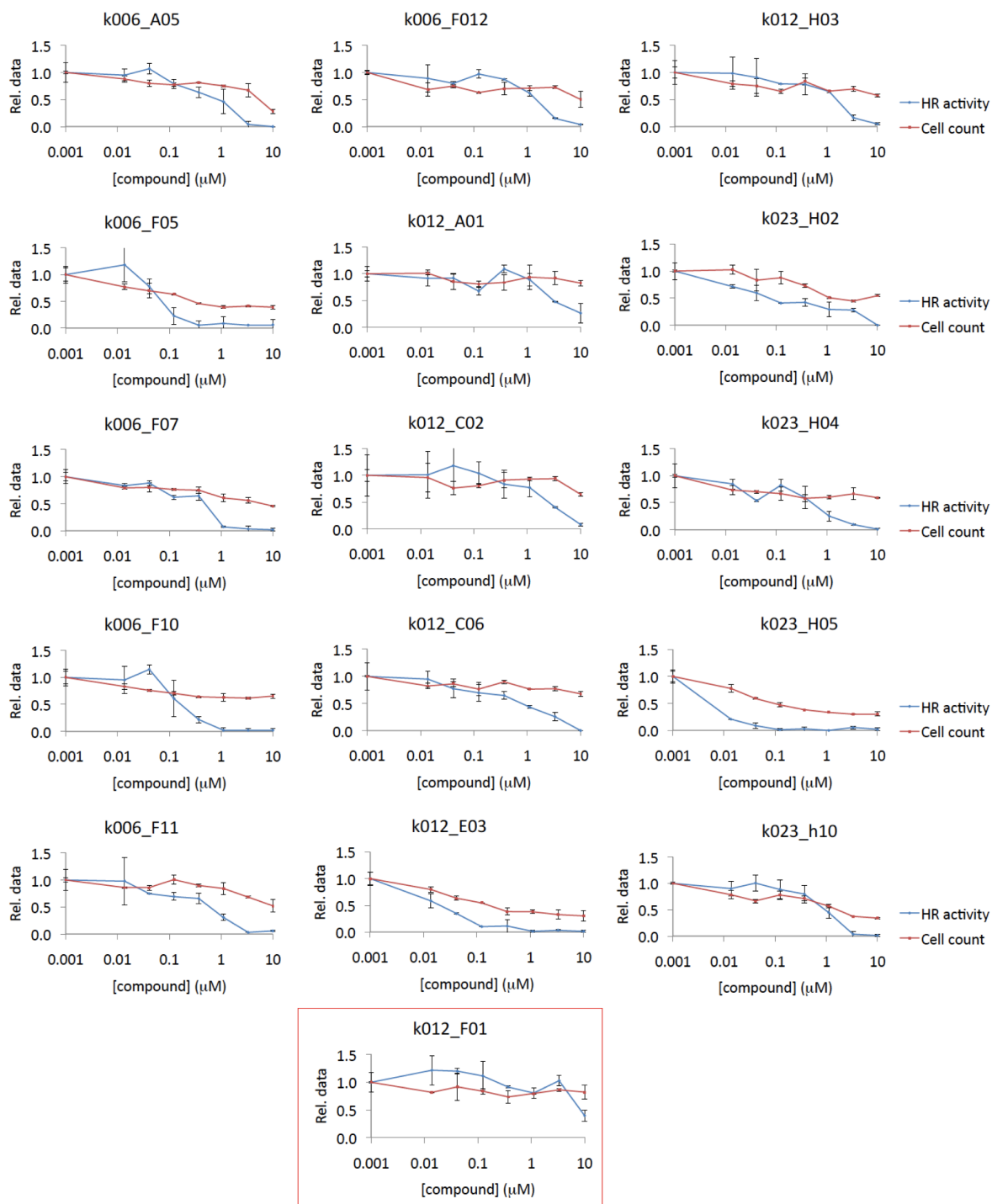
albeit exaggerated, to that seen in the siRNA screen (see Chapter 4). It is notable that those compounds causing a significant decrease in the percentage of GFP positive cells (potential HR inhibitors) did not yield such large errors, suggesting that the DR-GFP assay is suitable for the identification of prospective HR inhibitors in spite of the variability of the screen data. The Z-scores for all compounds in the library were calculated as described in Chapter 2 and provide a provisional hit-list of 19 compounds (Fig 5.1 (c)) for further study. It was also reassuring to observe that the screen did not have an obvious bias towards toxic compounds, as may be seen by the lack of a correlation between Z-score values for the preliminary hits and the cell count data collected during the screen (Fig 5.1 (d)).

Amongst the hit compounds were the CHK1/2 inhibitor AZD7762, a known radiosensitiser and HR inhibitor [141] and the HSP90 inhibitor Geldanamycin, the parent compound of the HR inhibitor 17-AAG [154]. These compounds serve as convenient internal positive controls, are annotated in Fig 5.1 and give confidence in the performance of the screen. In addition to these well characterised HR inhibitors, the preliminary hit list contained compounds active against kinases with potential roles in the DNA damage response. These included glycogen synthase kinase 3 (GSK-3) which has been shown to both activate p53 in response to IR as well as having a role in cyclin D1 destabilisation [213, 146] (see Chapter 1 for role of cyclin D1 in HR) as well as the PI3Ks and CDKs, inhibitors of which have been shown to have radiosensitising effects on cancer cells both *in vitro* and *in vivo* [102, 125, 116].

5.1.2 Kinase inhibitor screen: hit validation

Working with the preliminary hit list defined in Fig 5.1, hit validation was conducted by performing dose response assays in the DR-GFP assay for all preliminary hits (Fig 5.2). The purpose of this was twofold: firstly, any hit compound that did not produce a dose response cannot be considered as having a specific inhibitory effect on the assay and, secondly, I was concerned that a number of hits could be false positives arising from toxicity of the compounds. The data gathered from the FACS assay provides a cell count and allowed me to assess the toxicity of the compounds as well as their HR inhibitory effect. Thus, any compound providing a dose response, not purely driven by toxicity was considered a validated hit. The majority of preliminary hits (15/17) were validated in this way.

Figure 5.2 (*following page*): Dose response analysis in the DR-GFP assay in UGFP1 cells for hits identified from the kinase inhibitor screen. Blank normalised data are shown for both the DR-GFP assay and cell count data recorded in the same experiment. Relative DR-GFP activity is plotted in blue and relative cell count obtained by FACS in red. Those hits that produce a DR-GFP inhibition (HR inhibition) in excess of their toxicity (cell count) are plotted. The plot outlined in red shows an example of a compound (k012-F01) that does not produce DR-GFP inhibition in excess of its toxicity. Error bars represent the standard deviation of two replicates

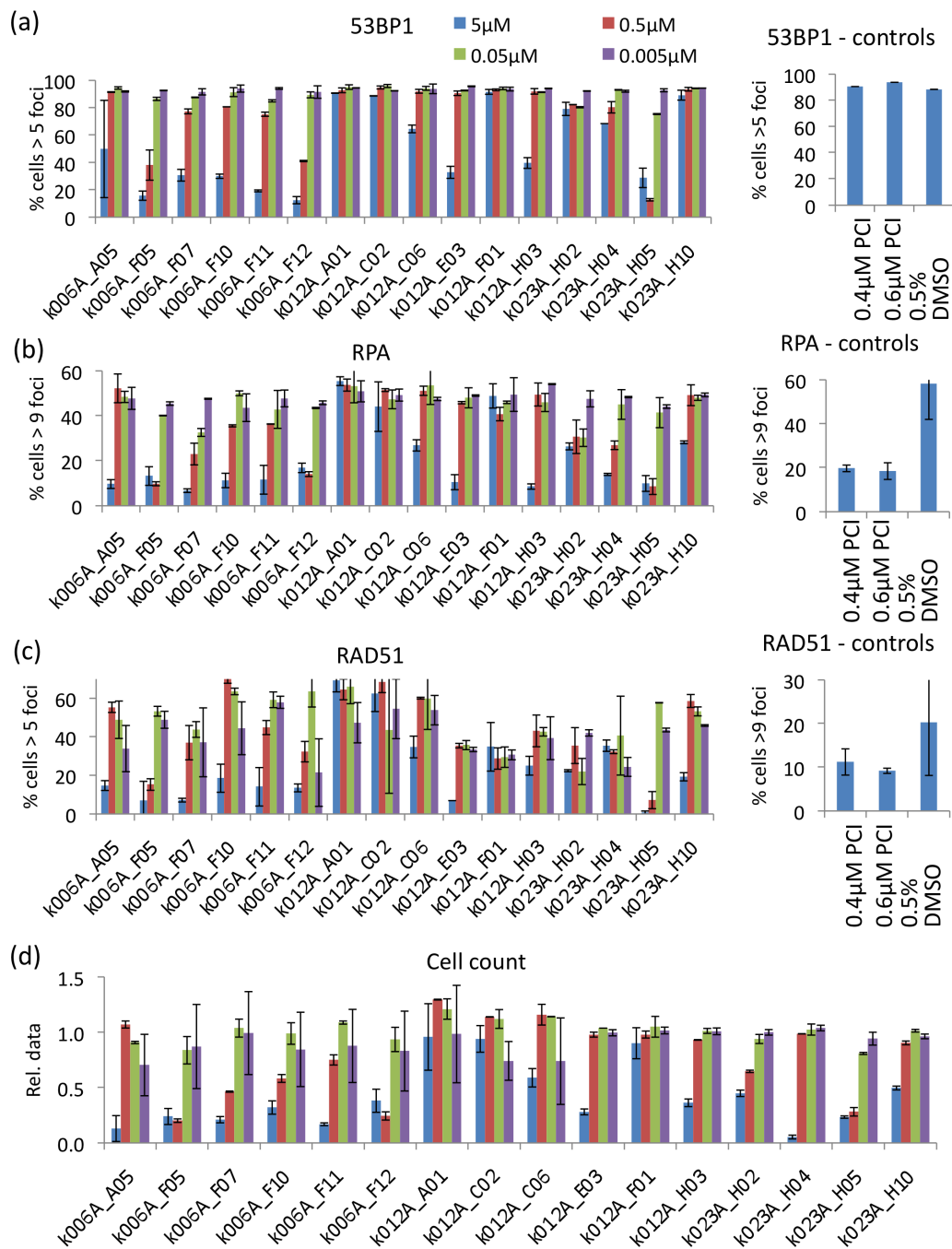


5.1.3 Kinase inhibitor screen: hit characterisation

DNA damage repair foci

Validated hits were now characterised at a range of concentrations (5, 0.5, 0.05 and 0.005 μ M) with respect to their effects on DNA repair foci formation following CPT treatment, specifically: 53BP1-, RPA- and RAD51-foci (Fig 5.3 (a)-(c)). In addition to the quantification of DNA repair foci, the InCell 1000 (GE) image analysis software was used to determine the cell count from the images used for foci analysis. This cell count data for each compound at each concentration was normalised to the cell count for DMSO treated cells to assess compound toxicity (Fig 5.3 (d)). The expectation was that a number of compounds, having been validated in the DR-GFP assay, would have an inhibitory effect on one of the foci readouts - although a compound that inhibits HR would not necessarily be expected to display a phenotype for the

Figure 5.3 (*following page*): Hit characterisation: foci formation in U2OS cells. Quantification of **(a)** 53BP1, **(b)** RPA and **(c)** RAD51 foci formation following hit compound treatment at different concentrations (see chart legend) together with their respective controls, run on the same 96-well plates. **(d)** Shows DMSO normalised cell count data for each compound, as scored using the image analysis software of the InCell 1000 automated microscope used for the foci image capture. Compounds were tested at multiple concentrations in all cases (see chart legend in (a)). Error bars represent the standard deviation of two replicates



selected molecular markers used in this study. A number of compounds were observed to exert an inhibitory effect upon foci formation assays, however, this was inseparable from their toxic effects in the foci assays in all cases, for example k006A-F05, k006A-F07, k006A-F10 and k006A-F11. This correlation between foci formation inhibition and toxicity suggests that the effects mediated by these compounds may be non-specific.

CPT sensitisation

In contrast to the foci formation assays, the CPT sensitisation assay (Fig 5.4 (a)), performed after 24 hour hit compound treatment and 120 hour continuous CPT treatment (50nM), showed a selection of potential HR inhibitors. Hit compound treatments both + and - CPT were normalised to their respective DMSO controls (+ and - CPT). Each hit compound + CPT value was then normalised to the corresponding hit compound - CPT value. Thus the normalised % cell viability value represents any sensitisation effect exerted by the hit compound. In some cases, compounds were found to be toxic and these data points have been excluded from the analysis.

Those validated hits that sensitise cells to CPT treatment (normalised % cell survival below 90%) have a diverse range of targets (Fig 5.4 (b)), from a broad spectrum of kinases to inhibition of protein kinase C (PKC), aurora kinases and PI3-kinases (Prof. S. Knapp, SGC, personal communication). Interestingly, two of the hits (K006-F05 and K006-F11) are close analogues and perhaps represent a pharmacophore with HR inhibitory activity, possibly

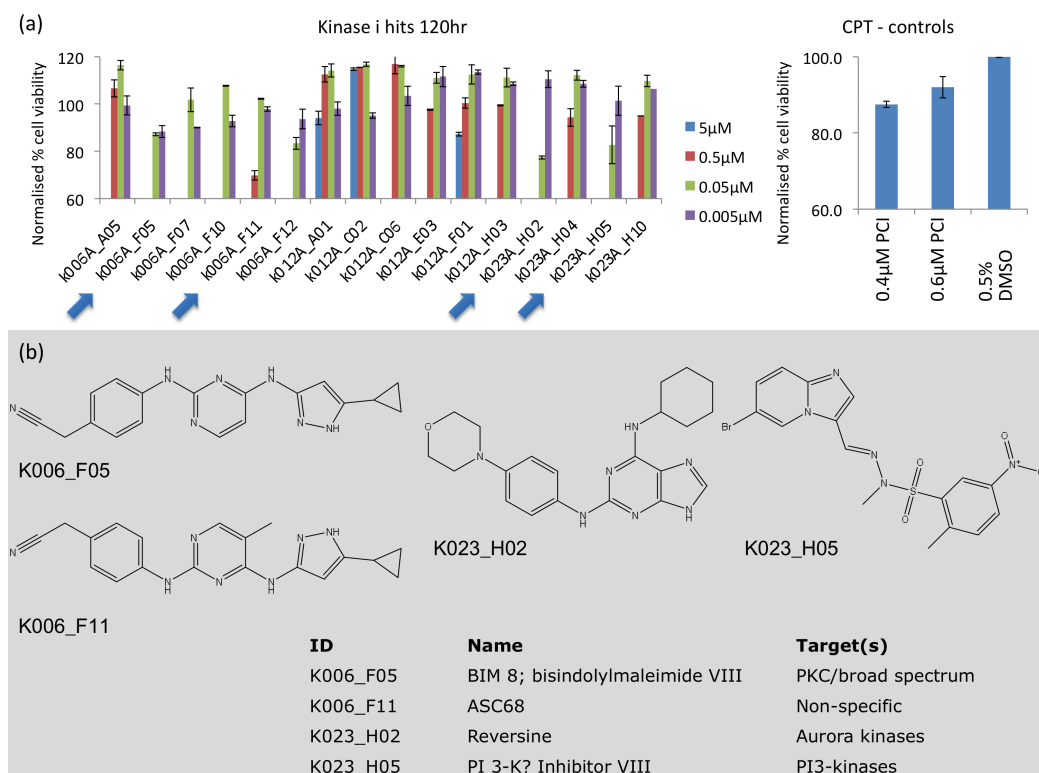


Figure 5.4: Hit characterisation: CPT sensitisation in U2OS cells. (a) DMSO and CPT normalised (see text in Results section for further details) % cell viability values for all validated hits from the kinase inhibitor screen. U2OS cells were treated with hit compound for 24 hours before a 120 hour continuous CPT treatment. Compounds were tested at multiple concentrations - see chart legend. Hits producing a sensitising effect (less than 90% normalised cell survival) are marked with blue arrows. Hit compounds at toxic concentrations are excluded from the chart. (b) Compound details of selected, validated hits. Error bars in (a) and (b) represent the standard deviation of two replicates.

via inhibition of the same target(s).

These data suggest a general role for kinase inhibitors in HR inhibition, which is in line with published work. Whether this general role reflects the promiscuity of the inhibitors used or the complexity of DNA damage signaling and the number of kinases that have an influence on HR is difficult to assess. Since none of the kinase inhibitors tested above had a pronounced effect upon the foci formation assays that was separable from their toxicity, it is difficult to identify how they may exert their inhibitory activity from the data I have collected.

5.2 NCI compound screen in the DR-GFP assay

The NCI compound libraries

In contrast to the focused kinase inhibitor screen detailed above, the second small molecule screen relied upon a chemical diversity approach to search for novel HR inhibitors. I opted to use the National Cancer Institute, NCI, diversity set compound libraries. These libraries comprise compounds that are not known inhibitors but have been selected from the NCI compound repository based upon their chemical diversity, in order to sample as much chemical space as possible whilst maintaining favourable properties for potential drug-like small molecule inhibitors. Properties selected for included the maximisation of the number of novel pharmacophores for each compound as

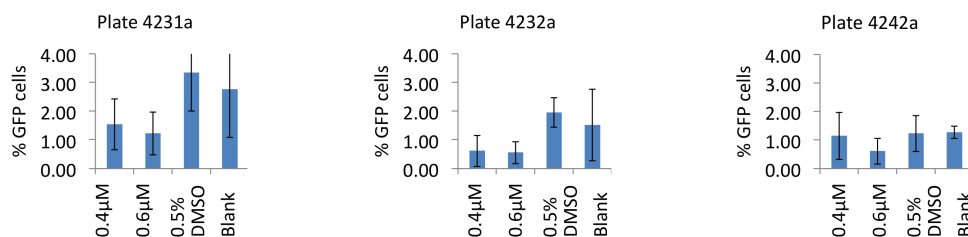


Figure 5.5: NCI compound DR-GFP screen in UGFP1 cells: control data. PCI, DMSO and Blank controls for three representative individual replicate plates of the three separate library plates shown in Fig 5.6 (three replicate plates a, b, c were made from each library plate). Each replicate plate contained the library compounds, plus the relevant controls. Error bars represent the standard deviation of three replicates.

well as limiting the number of rotatable chemical bonds to under six, giving a tendency towards planar, aromatic compounds with drug like characteristics such as cell penetrating ability and favourable hydrogen bond forming potential. The compound libraries used were the NCI diversity set I and NCI diversity set II, numbering 3440 compounds in total. Henceforth, these will be referred to as the NCI compounds.

5.2.1 NCI compound screen

Having completed the screen in the UGFP1 cells using the NCI compounds at a concentration of $5\mu\text{M}$ as outlined at the beginning of this chapter, initial data analysis allowed an assessment of the data quality in terms of the effectiveness of the positive controls (Fig 5.5) and the correlation of repli-

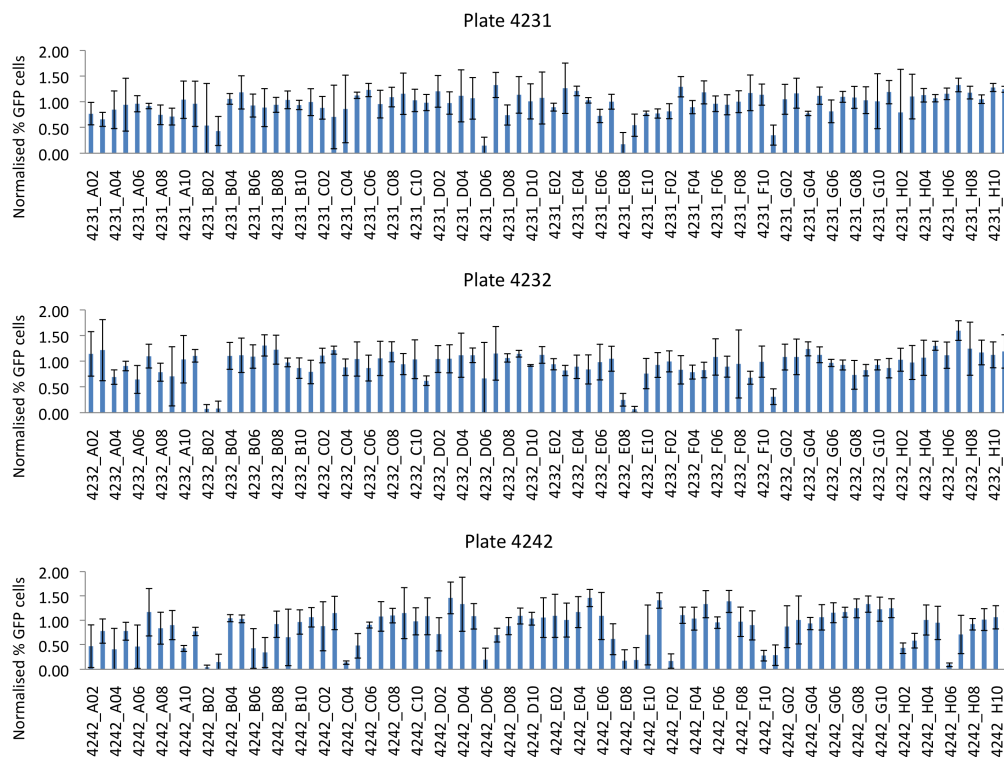


Figure 5.6: NCI compound DR-GFP screen in U2OS cells: screen data. Mean DMSO normalised % GFP cells for three library plates (each composed from three replicate plates). Each datapoint represents the mean of three values from three replicate plates. Error bars represent the standard deviation of three replicates.

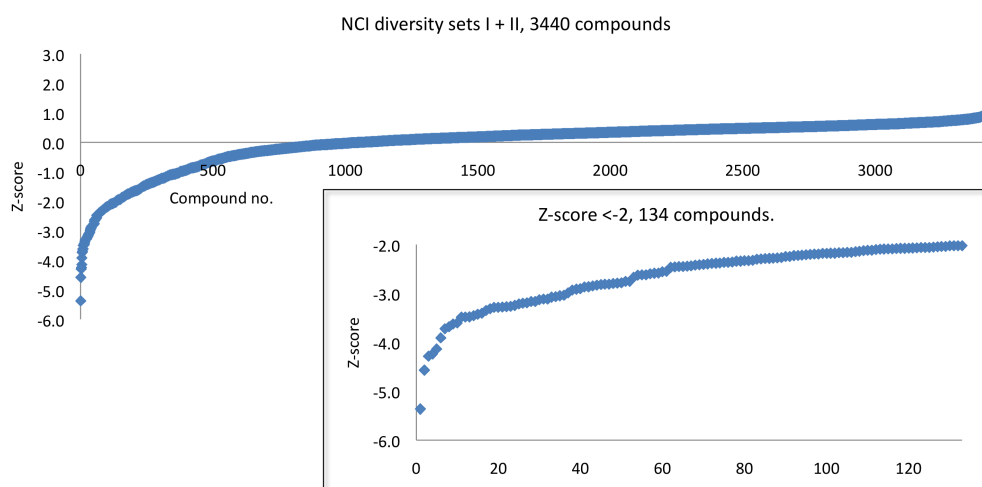


Figure 5.7: NCI compound DR-GFP screen in U2OS cells, full dataset: Z-scores for all 3440 NCI compounds screened using the DR-GFP assay. Preliminary hits (those with a Z-score ≤ -2) are shown inset.

cates (Fig 5.6). This analysis demonstrates that although the controls were generally effective, this was not always the case in all replicates of each compound plate (each compound plate was tested in three replicates and mean, DMSO normalised percent GFP cell data was plotted). Replicate data was of reasonable quality, especially for those compounds giving a decrease in the percentage of GFP positive cells. Although there was some concern over the control data, the full dataset was processed to avoid exclusion of any possible hits (Fig 5.7). Z-score analysis of the full dataset (Fig 5.7) shows a preliminary hit list of 134 compounds, or 3.9% of the library.

5.2.2 NCI compound screen: hit validation

All preliminary hit compounds still available (28 had discontinued by the NCI) were re-ordered as dry powders and dissolved in DMSO for validation and characterisation work. In contrast to the validation of the kinase inhibitor screen, I first decided to repeat the DR-GFP assay with all the hit compounds at the same concentration as the original screen ($5\mu\text{M}$) (Fig 5.8), as the number of preliminary hits was prohibitive for dose response analysis. The validation data showed that a significant number of compounds inhibited the DR-GFP assay upon repeat testing, though these were not in the majority. The replicate data for this experiment (Fig 5.8 (b)) was also of very good quality as compared to the original screen data. The number of false positives detected at this stage likely reflects the poor data quality of the initial screen as discussed above.

5.2.3 NCI compound screen: hit characterisation

DR-GFP dose response

With the hit validation process complete, a more manageable number of compounds (22) was selected for further work (those showing a significant reduction in the percentage of GFP positive cells in comparison to the DMSO control when retested in the DR-GFP assay, Fig 5.8 (a)). These selected hits were chosen for dose response analysis in the DR-GFP assay to identify those compounds that produced an HR inhibitory effect in excess of their toxicity (Fig 5.9). In the majority of cases (17/22), this turned out to be the

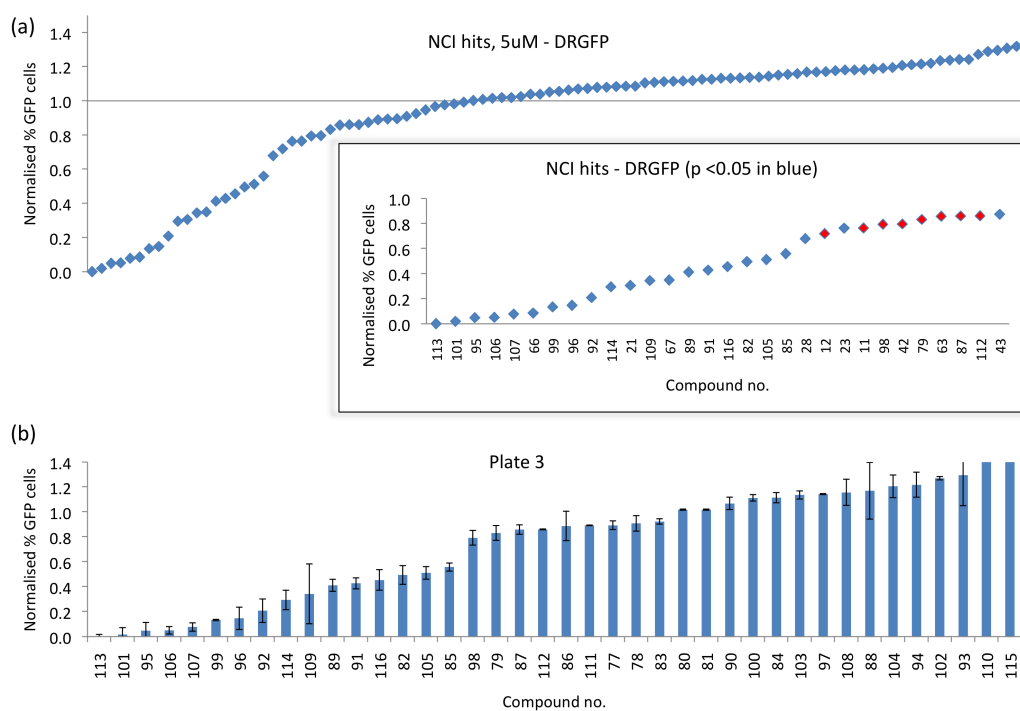


Figure 5.8: NCI hit validation. **(a)** Preliminary hits were retested at 5 μ M in the DR-GFP assay in UGFP1 cells and ranked according to their inhibitory effect, those causing a significant decrease in the percentage of GFP positive cells relative to DMSO treated cells (t-test, $p < 0.05$) are shown inset, in blue (t-test $p \geq 0.05$ in red). **(b)** Sample replicate data for one of the plates in the hit validation assay. Error bars represent the standard deviation of three replicates.

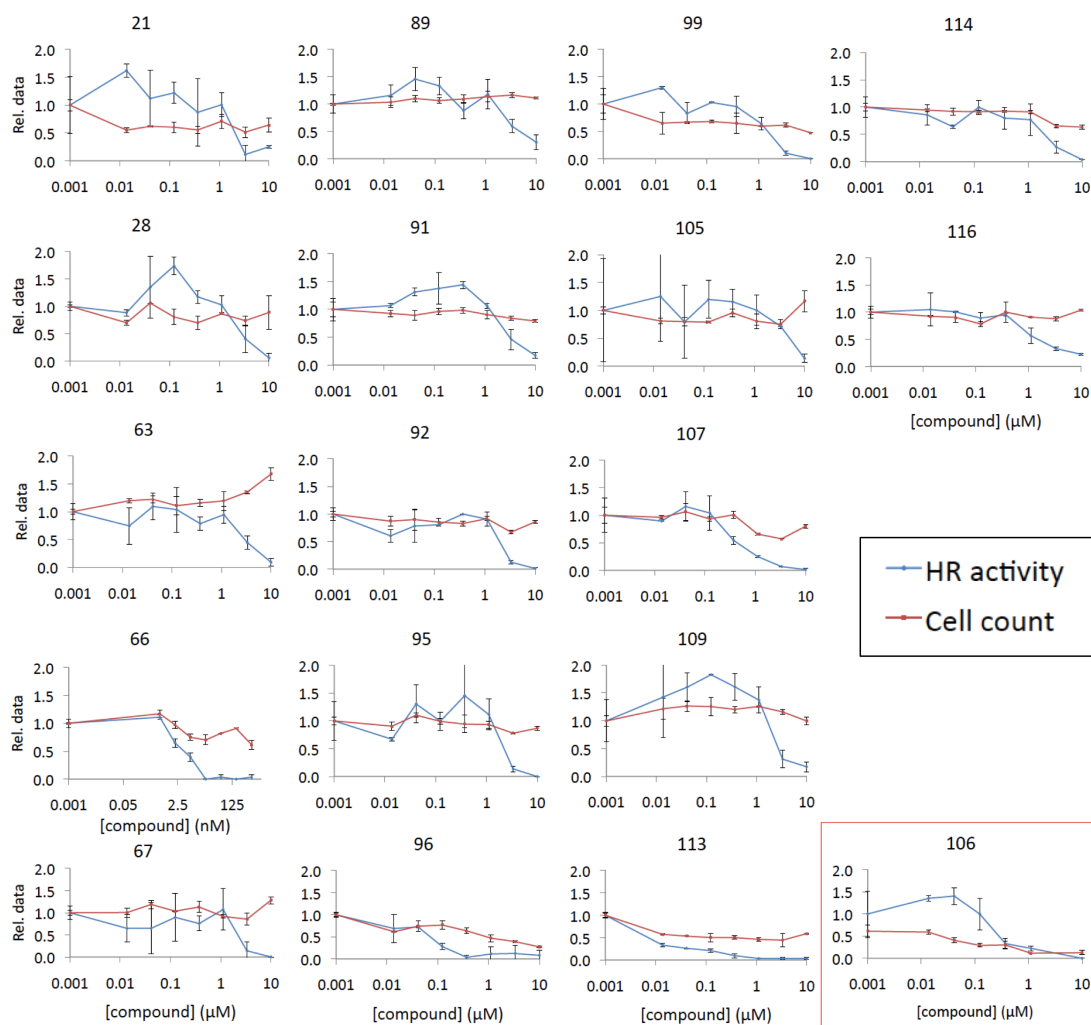


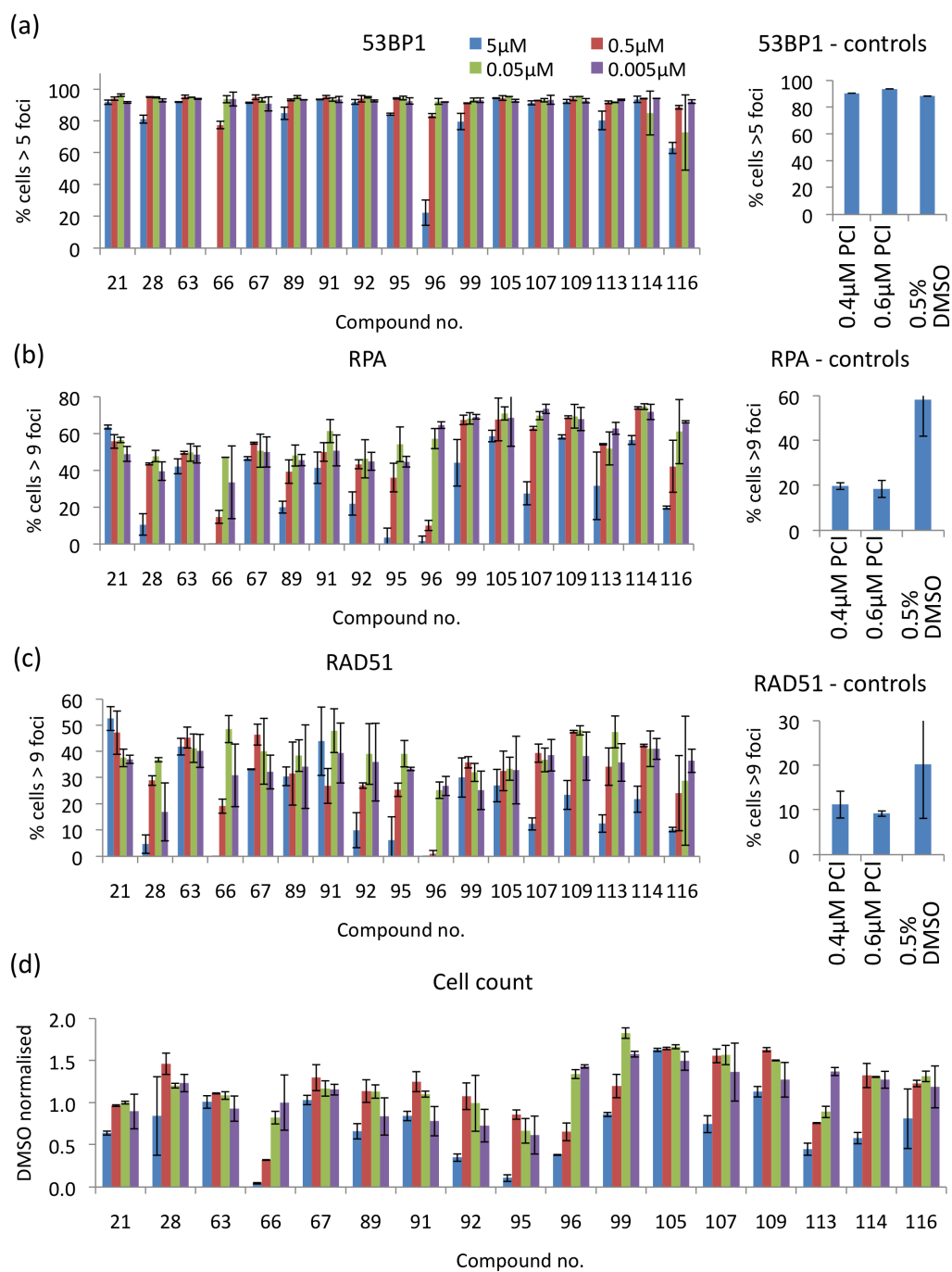
Figure 5.9: NCI hit characterisation: NCI hit compound dose response in the DR-GFP assay in UGFP1 cells. All plots show blank normalised data for both HR activity (percentage of GFP positive cells) in blue and living cell count data obtained by FACS in red. With the exception of the toxic compound 66 which was tested at 0-500nM, all compounds were tested at a concentration range of 0-10 μ M. A plot showing an example of a compound (106) not producing an HR inhibitory effect in excess of its toxicity is outlined in red. Error bars represent the standard deviation of two replicates.

case, lending confidence to both the initial screen data and the hit validation process.

DNA damage repair foci

Those hits that had been validated in the DR-GFP dose response assay, i.e. which have a specific inhibitory effect on the DR-GFP assay, were tested at multiple concentrations with respect to their effects upon DNA damage recognition and repair foci following DNA damage (CPT treatment) - Fig 5.10 (a). In general, none of the compounds impaired 53BP1 foci formation at non-toxic levels whilst two compounds (28 and 96) led to robust reductions of both RPA and RAD51 foci formation at non-toxic concentrations (Fig 5.12. These observations suggest that these compounds may have a specific HR

Figure 5.10 (*following page*): Hit characterisation: foci formation in U2OS cells. Quantification of **(a)** 53BP1, **(b)** RPA and **(c)** RAD51 foci formation following hit compound treatment at different concentrations (see chart legend) together with their respective controls, run on the same 96-well plates. **(d)** Shows DMSO normalised cell count data for each compound, as scored using the image analysis software of the InCell 1000 automated microscope used for the foci image capture. Compounds were tested at multiple concentrations in all cases (see chart legend in (a)). Error bars represent the standard deviation of two replicates Error bars represent the standard deviation of two replicates.



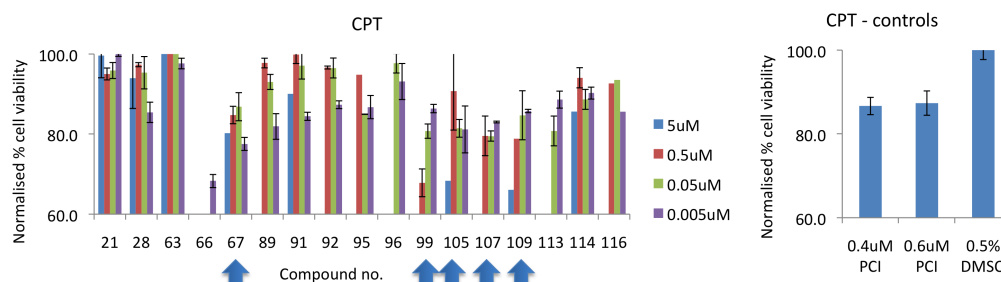


Figure 5.11: Hit characterisation: CPT sensitisation for validated hits from the NCI compound screen in U2OS cells. DMSO and CPT normalised (see text in Results section for further details) % cell viability values for all validated hits from the NCI compound screen. U2OS cells were treated with hit compound for 24 hours before a 120 hour continuous CPT treatment. Compounds were tested at multiple concentrations - see chart legend. Hits producing a sensitising effect (less than 90% normalised cell survival) are marked with blue arrows. Hit compounds at toxic concentrations are excluded from the chart. Controls are shown on the right. Error bars represent the standard deviation of two replicates.

inhibitory effect acting downstream of DNA damage recognition but before DNA resection or RPA loading and may account for the observed DR-GFP assay inhibition exerted by these compounds.

CPT sensitisation

Validated hits from the NCI compound screen were next tested for their ability to sensitise cells to CPT treatment (Fig 5.11 - compounds at toxic concentrations were excluded from the results). The majority of compounds

did not sensitise cells to CPT-induced damage, although compounds 67, 99, 105, 107 and 109 did display a more notable sensitisation activity (<90% normalised cell survival). It was expected from the foci formation data that compounds 28 and 96 would sensitise cells to CPT, however such an effect was not observed (Fig 5.12). Nonetheless, these compounds represent the most interesting for follow-up work as further experiments may be guided by the initial observations taken from the foci formation assays.

The results of the sensitisation assays during the course of hit characterisation for both the kinase inhibitor and NCI library compound screens suggest that the sensitivity of this assay may not be as high as desired. Only modest sensitisation effects are seen with the positive control compound, although this may be due to the lack of specificity of PCI-24781 as an HR inhibitor [94, 15]. Further work on those compounds showing inhibitory activity in the DR-GFP assay and either an inhibition of repair foci formation or sensitisation to CPT treatment is warranted to substantiate the initial findings. Reducing the number of compounds from the number used through the initial validation process for such follow up experiments will open up new directions in compound characterisation as focus can shift from high-throughput to data quality and assay sensitivity and specificity.

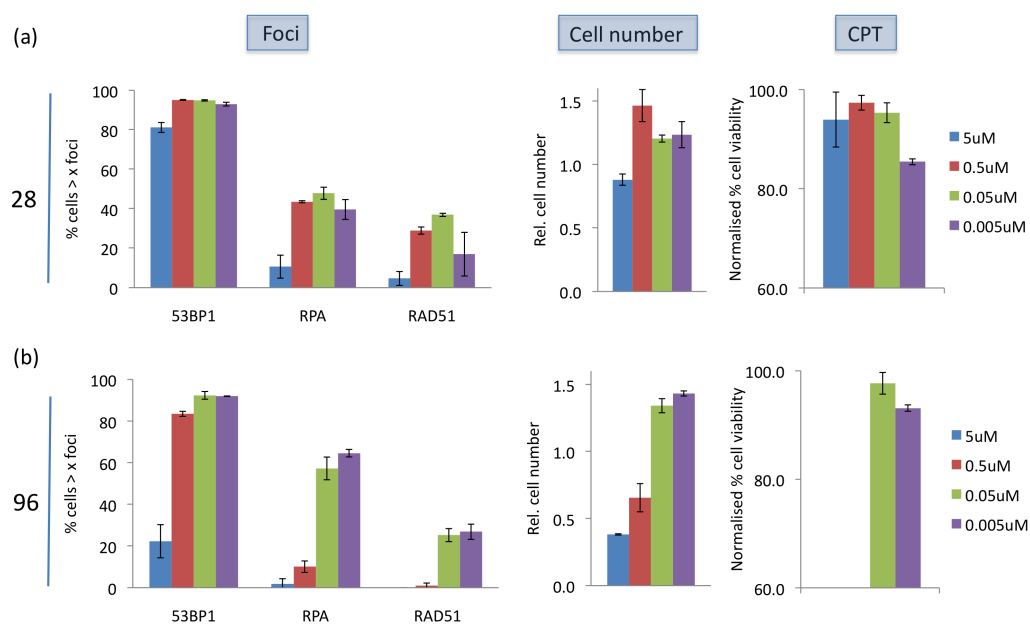


Figure 5.12: NCI compound screen: hits. The effect of these compounds on foci formation in U2OS cells, together with the normalised cell number data for the foci experiments are provided along with the CPT sensitisation data. **(a)** compound 28 **(b)** compound 96. Error bars represent the standard deviation of two replicates

5.3 DR-GFP small molecule screens: summary

In spite of the drawbacks of the DR-GFP assay with respect to small molecule screening previously discussed (Chapter 3), I successfully employed the assay to screen almost 4000 compounds leading to the identification of candidate HR inhibitors. Following completion of the kinase inhibitor and NCI small molecule screens together with the validation and subsequent characterisation work conducted on a large number of preliminary hit compounds, it is possible to define a reduced number of hits for future work. These prospective HR inhibitors represent compounds that have demonstrated inhibition of either (i) the DR-GFP assay together with RPA and RAD51 foci formation assays or (ii) the DR-GFP assay together with sensitising cells to DNA damage induced by CPT.

Whilst it may not be expected that compounds that inhibit the DR-GFP assay should necessarily inhibit RPA and RAD51 foci formation, it is surprising that those that do show such an effect (NCI compounds 28 and 96) do not also exhibit a sensitising effect to DNA damage by CPT, although HR is not the sole pathway which may repair CPT induced damage - see Discussion. Further, more detailed work on this discrepancy is required to be able to assert with confidence that these compounds are HR inhibitors. A more suitable approach to firmly establish whether or not these compounds produce a sensitising effect to DNA damaging agents, made possible by re-

Table 5.1: Summary of small molecule screen hits with potential HR inhibitory activity. Those compounds from each small molecule screen that inhibit both the DR-GFP and RPA- and RAD51-foci formation assays and those that inhibit both the DR-GFP assay and sensitise cells to CPT treatment are listed below

Screen	DR-GFP and RPA-, RAD51 foci formation	DR-GFP and CPT sensi- tisation
Kinase inhibitor	-	k006A-F05, k006A-F11, k023A-H02, k023A-H05
NCI	28, 96	67, 99, 105, 107, 109

fining the number of hit compounds down to a more manageable number, could be provided by clonogenic survival assays. Such assays should test the response of hit compounds, at multiple concentrations, against a spectrum of DNA damaging agents opening up a wider experimental window in which to detect a sensitising effect.

My follow-up work attempted to address the issue of working with a range of compounds all of which produce a phenotype at different concentrations. Further work on the refined hit list would benefit from full dose response profiles of each compound in all assays, to better define a suitable working concentration. Such data should allow the separation of specific HR inhibitory effects from the general toxic effects of any given compound.

Chapter 6

Discussion

It is helpful at the start of this chapter to bear in mind the original project aims as the context within which the discussion of my results will take place, these were:

1. The identification of novel proteins involved in homologous recombination.
2. The identification of small molecule inhibitors of homologous recombination.

6.1 Assay development

6.1.1 The DR-GFP assay

Initial assay development

My initial work centred upon optimisation for high-throughput of the DR-GFP assay in MRC5 cells with a stably integrated DR-GFP construct (MGFP). Having demonstrated that the assay was functional and that I was able to modulate its activity by knockdown of known HR proteins (Fig 3.1), it was important to begin to optimise the assay conditions to gain maximum sensitivity for future screening work. Whilst siRNA transfection conditions were defined to give strong protein knockdown with a minimum amount of siRNA (minimising possible off-target effects), assay sensitivity was improved by clonal selection providing the MGFP-derived clone MGFP6 for further work, see Fig 3.2.

For the siRNA screen, I opted to first establish effective protein knock-down over 48 hours, before inducing a double strand break in the DR-GFP reporter by I-SceI transfection. I believe that this approach is likely to yield the greatest phenotype in this assay as any proteins required for HR will be at reduced levels from the start of the damage recognition process through to completion of repair. This expands the spectrum of hits that may be anticipated to include proteins involved at early stages of the DNA damage recognition and HR processes. The timescale of HR mediated DSB repair lies within the 48 hour timescale of the DR-GFP assay as applied during my

work, with the majority of HR dependent HR repair occurring between 4-24 hours after damage [58, 91].

Having defined the siRNA assay protocol and as my work with the MGFP6 cells continued, concern mounted over the sensitivity of the assay in this cell line, with only marginal effects seen following XRCC3 knockdown (XRCC3 knockdown MGFP cells still demonstrated approximately 60% of the NT siRNA treated cells HR activity as measured by the DR-GFP assay, (Fig 3.1)). It was difficult to envisage that with such a small phenotype observed after knockdown of a core HR protein [165] involved in the loading of RAD51 onto resected DNA [225] as well as downstream of RAD51 loading, possibly as part of the HJ resolvase machinery [118], that I could expect to identify novel HR proteins. Fortunately, I had access to a clone of the U2OS-DR-GFP cell line (UGFP1) that had been prepared by Dr C. Lundin within our laboratory and which yielded more favourable results with the protocol developed for the MGFP6 cell line. siRNA knockdown of protein levels, as measured by Western blot, was equally effective in both cell lines (Figs 3.4 and 3.6) and standard plasmid transfection protocols consistently and effectively used in our laboratory were also used in both instances. Given the different derivations of the two cell lines, it is possible that the already unstable genetic background of the p16 null U2OS cancer cell line as compared to the primary MRC5 cells render it more sensitive to perturbation of DNA repair processes. It is possible that U2OS cells are more amenable to transfection, but the % GFP response generated in the MGFP6 and UGFP1 cells following

I-SceI transfection (although variable in both cases) was comparable.

Evident throughout all my work is the variability of the efficiency of GFP induction from experiment to experiment. This could be seen by the variation in the % GFP cells both from experiment to experiment as well as between different plates from the same screen (run at the same time), see Figs 3.6 and 4.2 for examples. It was also clear that there could be a large well to well variation in % GFP cell induction under identical conditions (Fig 3.7). Variability in the performance of the DR-GFP assay is consistent with observations in the literature, with induction of GFP in different studies ranging from <1% to >5% in some studies using hamster cells [1, 165] to 10-15% in human cancer cell lines [172] with relatively high variation between replicates not uncommon. Although the experiment to experiment variation did not present a specific problem, as data that required comparison (such as the screen data) was always normalised to the negative control (NT siRNA or DMSO vehicle dependent on the assay), it warrants some explanation whilst the well to well, intra-experimental variation must be accepted as an inherent drawback of the DR-GFP assay. There are a variety of possible causes for the observed variation: firstly, I-SceI transfection efficiency is unlikely to be identical in all experiments, perhaps due to small differences in cell density of each well of a 96-well plate that are difficult to eliminate when handling large numbers of samples. Furthermore the kinetics of GFP induction may not be identical in every experimental repeat. It was noted that GFP induction continued to rise 48 hours after I-SceI transfection (the experimental

endpoint), therefore small changes in the kinetics of GFP induction between different experiments could result in different levels of induction at identical timepoints. Furthermore, when processing multiple plates, especially for the screens, it was difficult to ensure exactly 48 hours between I-SceI transfection and cell trypsinisation. A final potential cause of inter-experiment variation was the difference in GFP induction between early and late passage cells as noted when comparing the kinase inhibitor screen (Fig 5.1, conducted with late passage UGFP1 cells) with the siRNA screen at 6nM (Fig 4.2, conducted using low passage UGFP1 cells). This signal decay may arise from outgrowth of cells within the population which had acquired mutations rendering the DR-GFP construct non-functional. Throughout my work, cells were used over the course of 2-3 months until the GFP induction began to noticeably drop. An obvious solution is to thaw out low passage cells more regularly, but with such a long timecourse for the project, this risks depleting cell stocks at an undesirable rate.

DR-GFP assay: technical issues relating to high-throughput screening

A recurring problem with the DR-GFP assay was the high sample-to-sample variability witnessed in most experiments. There are a number of explanations for this observation. Firstly siRNA-mediated protein knockdown is not 100% efficient, coupled with the less than 100% efficiency of I-SceI transfection, one cannot assume that all cells with established protein knockdown

will be transfected with I-SceI. This inherent drawback of the assay is difficult to overcome using the siRNA approach. I attempted to account for the variability of I-SceI transfection by performing a co-transfection of I-SceI with DsRed. Levels of red fluorescence detected by FACS at the endpoint of the assay could then be taken as a measure of transfection efficiency and the data normalised to take this into account. I found a poor correlation between red fluorescence and green fluorescence when testing this approach (Fig 3.7), suggesting that it was not an appropriate strategy to employ. In hindsight, cloning of dsRed into the same vector as I-SceI may have provided a solution to this problem.

A further, insurmountable problem with the assay was the inefficient harvesting of the cells for FACS. A higher cell number at the assay endpoint provides more robust data given the low recombination efficiency of the DR-GFP assay (varying from around 2-5%). Maximising the cell number (to >1000 cells) relied upon effective trypsinisation, thorough resuspension of cells and efficient cell pick up by the automated FACS machine high-throughput sampler. These technical problems are set in the context of a population of cells which have undergone two transfections, each of which reduce cell viability. Both siRNA and I-SceI transfections also needed to be performed under optimum conditions whilst providing a maximal cell number. Although the siRNA reverse transfection could be performed at relatively high cell number (12000 cells per well in 96-well plate) with little effect on the results, the I-SceI transfection is of reduced efficiency if the cells are overconfluent. Growing

the cells to subconfluency (<80% confluent) for the I-SceI transfection was likely to yield too few cells to give reliable data at the end of the assay. An inherent limitation which applies to all high-throughput screens, is the inability to identify and rectify possible problems during the experiment due to the sheer volume of samples being processed, especially when using a robotic platform that, once running, cannot be adjusted through the course of an experiment. In summary, the DR-GFP assay possesses a number of known limitations which are compounded when processing numerous 96-well plates in a single run, as was the case for the screen. An additional limiting factor for assay throughput was the plate read time of approximately 45 minutes, producing a significant bottleneck in the screening process. One possible solution to the problems imposed by the FACS assay would be to measure the GFP fluorescence per sample *in situ*, for example using an automated spectrophotometer. I attempted this but found the GFP signal to be too weak for detection.

During the course of my work, a genome wide DR-GFP RNAi screen was published [182]. It is worth pointing out some of the differences between the approach used in this screen in contrast to mine. In contrast to my approach of siRNA mediated protein knockdown (48 hours) and subsequent DSB induction (I-SceI transfection) followed by 48 hour incubation in advance of FACS, the authors [182] elected to establish protein knockdown at the sametime as DSB induction. I believe that this alternative strategy is more likely to lead to a low hit-rate than my approach as one cannot en-

sure full knockdown of a target protein before the DSB is induced and the damage recognition and repair process is initiated. The logistics of conducting a genome-wide screen may have dictated that the authors used this less time-intensive approach. A further difference between the published DR-GFP screen and my own work was their use of 384-well plates as opposed to 96-well. The endpoint of the assay in both cases was FACS based and I believe the problems discussed above relating to efficient retrieval of cells would be exacerbated in 384-well plate. My own work showed how difficult it was to obtain any reliable data using the DR-GFP assay in 384-well plate (Fig 3.10 (d)).

The small molecule screen suffered from fewer technical drawbacks than the siRNA screen on account of the relative simplicity of a drug treatment as compared to siRNA transfection. It is, however, important to note a key difference between the two protocols that may have a bearing on the results obtained. Whereas the siRNA screen established protein knockdown in advance of I-SceI transfection, the small molecule screen necessitated treatment with the small molecules at the same time as I-SceI transfection. This approach was dictated by the time required to screen 3440 compounds in triplicate in a 96-well format, a total of 45 compound plates, representing 135 cell plates in the final experiment. The specification of the robotic handling station meant that no more than 12 plates could be processed in a single run, which (together with compound preparation) took most of a working day to complete. In order to complete the screen in as short a time as possible, with

the same stock of cells, a minimal assay timescale was desirable.

A final consideration when using the DR-GFP assay is that any disruption of transcription, translation or protein maturation may yield a false positive result. Awareness of this risk was essential for the follow up work for both my screens. Proposed modifications to the DR-GFP assay and feasible alternatives are discussed later in this chapter.

6.1.2 Synthetic lethal screen

With the limitations of the DR-GFP assay, especially with relation to small molecule screening as described above, I was aware that a higher throughput approach may be required. To this end, I assessed an alternative approach relying on the predicted sensitivity of PARP1 knockout MEFs to HR inhibition based upon the reversal of the well described synthetic lethality displayed by HR deficient BRCA1 and BRCA2 cells treated with PARP inhibitors [19, 48]. I established that just such a synthetic lethality appeared to exist with the specific killing of PARP1-knockout MEFs (A11 MEFs) relative to their isogenic wild type counterparts (A19 MEFs) by HR inhibition, as induced by the HDAC inhibitor PCI-24781 (Fig 3.8 (b)). Based upon this observation I tested the drug sensitivity screen in 96-well plates, with cell viability endpoint comparing A11 and A19 MEF viability. This simple endpoint required only an hour incubation with resazurin dye followed by fluorescence analysis on an automated, high-throughput plate reader (approximately 15 seconds per plate) and was accordingly better suited to a high-throughput strategy

than the DR-GFP assay. However, the phenotypic instability of these cell lines with the gradual diminishment of the initially observed sensitivity of A11 PARP-knockout MEFs to PCI-24781 treatment as a function of cell passage number (Fig 3.9 (b)) led me to halt this screening approach.

6.2 DR-GFP assay: improvements and alternatives

6.2.1 DR-GFP assay improvements

There are a number of possible technical improvements that could be applied to the initial protocol. Some were applied throughout my studies and others may have been of use with the benefit of hindsight. A systematic error in the DR-GFP assay that I believe was one of the main reasons for the high variability witnessed throughout my work was absence of controls for the I-SceI transfection. Consistent transfection efficiency across all samples reduces the noise of the resulting dataset. Whilst it is impossible to ensure that every well in a 96-well plate is transfected with identical efficiency, normalisation of results to an internal transfection control could account for this deficiency. Although this was attempted by cotransfection of I-SceI with a DsRed plasmid, a more suitable approach would be to design a plasmid bearing both the I-SceI and DsRed sequences, possibly separated by an internal ribosome entry site. This approach was not investigated but I believe it would provide a transfection efficiency readout for every well to which the percentage of

GFP positive cells could be normalised. Such a system, as well as improving data quality, would also allow identification of false positive hits that mediate their effects through either an inhibition of the transfection process (a likely problem for the small molecule screen) or through inhibition of the transcription, translation and protein maturation of the recombined GFP generated through functional HR.

A problem which was addressed over the course of this project was the trade-off between transfection efficiency and toxicity with the transfection reagent PEI, causing 10-20% toxicity (PEI was used for assay optimisation and for the screen on account of its low cost). In order to maximise the response of the DR-GFP assay, a concentration of PEI had to be used that was toxic to the cells, thus setting up a compromise between the dynamic range of the assay and cell number available for FACS analysis, which could be as low as 1000 cells. Later experiments were conducted using Lipofectamine 2000 as a transfection reagent, this proved less toxic and more efficient with respect to induction of fluorescence in the DR-GFP assay.

A final technical issue addressed during the course of my work was the cell harvesting for FACS. My early studies had exploited the high-throughput setting of the FACS machine, requiring a total well volume of 10 μ l or less of trypsinised cells. Although quick, this setup was very inefficient at retrieving all cells from each well. Accordingly, the volume of trypsinised cells was increased to 60 μ l for the screen, greatly improving cell pick up efficiency and data quality whilst maintaining plate read times of under an hour. The

post-screening follow-up work presented here further increased the volume of trypsinised cells to 130 μ l which gave an incremental improvement in cell pick-up efficiency and data quality. Such a high well volume would have proven too slow for the screen, with a plate read time of around 90 minutes.

siRNA screen

A source of experimental variation for all siRNA work is the differential knockdown efficiency between different wells of a 96 well plate. When considering the screen, some of this variation may arise from pipetting variation between wells. A possible solution, for which we did not possess the tools at the time, is to use pre-plated siRNA and transfection reagent to which cells are directly added. Whilst such an approach relies on high quality reagents from the supplier, it simplifies the task of the researcher and is more amenable to the handling of large numbers of plates with a relatively basic robotic handling station, such as the one used in this work. Such an approach has recently been adopted in our laboratory (unpublished work) with favourable results in terms of ease of use and data quality, but was not available at the outset of my project.

Small molecule screen

As has been suggested above, it is possible that the small molecules used in the screens could lead to false positive results by inhibition of the transfection process. Although the small molecule and I-SceI cotreatment was designed to reduce the complexity and timescale of the screen, a possible alternative

would have been to treat cells with compounds in the morning and then wash the plates and transfect with I-SceI in the afternoon. Such an approach would not greatly increase the complexity of the protocol and would maintain its overall timeline but may reduce the return of false positive results. A possible drawback would be the limited exposure time of cells to each compound, though within 3-4 hours it may be expected that cell-permeable compounds have been able to hit any prospective targets.

There are further considerations to be made with small molecule screens. Firstly, is continuous exposure of the cells to each compound necessary to demonstrate a phenotype, as opposed to an acute treatment? This is likely to depend on the target of the compound and its interaction kinetics. Compounds that bind tightly to their targets and demonstrate slow dissociation kinetics are likely to yield a phenotype after an acute treatment, whilst those that are less potent and dissociate more swiftly from their targets will require a continual treatment to mediate any observable effects [208]. Secondly, is pretreatment with a compound before I-SceI transfection desirable? This is likely to be the case, as pretreatment allows for the compound of interest to permeate the cell membrane and reach its site of action allowing establishment of target inhibition before the damage recognition and repair machinery is activated by I-SceI transfection.

A problem encountered with many small molecule screens is the assumption that the desired phenotype may be detected at only a single concentration of compound. Many screens use only a single concentration for practical

purposes [108, 99, 43, 2] and my screen was no different. However, with suitable throughput capacity, it is far more desirable to screen compounds at multiple concentrations. Hits are then selected as those compounds demonstrating a dose response, giving a more refined preliminary hit list for further work as well as providing initial information on the concentration range of each hit compound that should be used in downstream validation and characterisation assays.

In light of these considerations, but bearing in mind the throughput of the DR-GFP assay, a modified small molecule screening protocol may be suggested. Plated cells are first treated with compound for 6 hours before washing of cells and I-SceI transfection. Following overnight incubation with the transfection reagents (sufficient time for DNA to enter the cell), compound treatment would then be repeated, with the cells continually exposed to the compounds until the assay endpoint. I believe such an approach may both reduce the number of false positives detected and increase the window of detection for less potent inhibitors.

6.2.2 Alternative assays

Throughout my work I have used different assays to characterise HR deficiency, these may be scaled up for high-throughput and could be considered as alternatives to the DR-GFP assay used in my screens.

DNA damaging agent sensitisation

Perhaps the most straightforward is screening for those siRNAs or small molecules that sensitise cells to treatments that induce DSBs, such as CPT or IR. Such screens benefit from their inherent simplicity, requiring only a DNA damaging treatment and an appropriate cell viability assay as a high-throughput endpoint. Such sensitisation assays do, however, suffer from a lack of specificity. This arises from the possibility that those sensitising effects detected may arise from non-DNA repair mediated effects such as the inhibition of apoptosis, or in the case of a small molecule screen, any compound that inhibits the metabolism or removal from the cell of a DNA damaging drug such as CPT.

A potential problem with a simple DNA damage inducing agent sensitisation screen is the specificity of the chosen drug and the DNA repair pathway(s) employed to repair the induced damage. During the course of my work, I used CPT along with other DNA damaging agents to help characterise siRNAs or small molecules that may inhibit HR. CPT treatment leads to poisoning of Topoisomerase I and subsequent replication fork stalling [78]. The persistent SSBs induced by CPT poisoning and trapping of Topoisomerase I in complex with DNA may be converted to DSBs by collision with an ongoing replication fork [191]. Such replication dependent DSBs are major substrates for HR [163]. However, repair of CPT induced stalled replication forks may also proceed via stalled fork regression mediated by members of the RECQ family of helicases, BLM [34], RECQL4 [158] and WRN [30]. In

light of the complexity of the response to CPT induced damage it is important to consider for my work that any observed CPT sensitisation may arise from inhibitory effects on pathways other than HR and furthermore, that CPT sensitisation is not specific for HR inhibition. This concern was addressed during the siRNA screen follow-up process with sensitisation to multiple DNA damaging agents (CPT, MMC and IR) tested. In retrospect, an agent more suitable to the induction of damage that more specifically requires HR for repair would be the DSB inducing agent bleomycin. However, DSBs may be repaired by both HR and NHEJ, so caution would still be required with bleomycin sensitisation experiments.

Immunofluorescence

An additional alternative is an immunofluorescence-based foci formation screen already successfully applied in both RNAi and small molecule screening [103, 98]. Whilst we have the capacity in our laboratory for high-throughput immunofluorescence data collection and analysis using the InCell 1000 automated microscope (GE), such an assay also suffers from potential drawbacks. A foci-formation assay can provide information focusing only on one step of the complex and multifaceted HR pathway. This is in contrast to the DR-GFP assay that can give a readout for perturbation of HR at any step. A foci formation assay is also relatively complex to perform on a large scale, with a number of possible stages where errors may be introduced, such as DNA damage induction, staining with the primary antibody and staining

with the secondary antibody and appropriate blocking. Nevertheless, such assays have been used successfully to identify novel DNA damage repair proteins such as 53BP1-foci formation in the discovery of the RIDDLE syndrome protein RNF168 [188].

6.3 siRNA screen

6.3.1 Assay performance

The siRNA screen in UGFP1 cells was conducted twice at an siRNA concentration of both 10nM and 6nM. The reduction of the siRNA concentration to 6nM was necessary to counteract the off target effect of NT siRNA upon the DR-GFP assay at higher concentrations (Fig 3.6). Assay performance was assessed on the basis of the effectiveness of the controls and the quality of the replicate data (each siRNA was tested in triplicate) [Figs 4.1 4.2 4.4]. Whilst the RAD51 siRNA positive control in both screens was clearly effective in reducing the percentage of GFP positive cells to almost background (minus I-SceI) levels, it was also immediately clear that the NT siRNA at 10nM was having a negative effect on the percentage of GFP positive cells as compared to the blank control (cells treated with transfection reagent only). This off-target effect reduced the window within which siRNAs inhibiting HR could be detected which, coupled to the generally low induction of GFP expression that is characteristic of this assay, led me to repeat the screen at 6nM of siRNA.

The 6nM siRNA screen showed a diminished off-target effect of the NT siRNA and as a result of using low passage UGFP1 cells, the level of GFP induction increased and replicate data quality improved. It was consistently observed throughout my work that freshly thawed cells bearing the DR-GFP construct (both U2OS and MRC5 cell lines) gave a higher induction of GFP and generally better data quality than later passage cells.

As a result of the changes implemented after the initial 10nM screen, I now had two full screen datasets. As has been described above, there are a number of inherent drawbacks with this screen and it was therefore an advantage to have two datasets to increase the pool from which potential hits were to be drawn. The correlation between the datasets was not strong, especially in the region where I was expecting to identify hits. This may be accounted for not only from the different assay conditions between the two screens, with possible differences in protein knockdown efficiency across all siRNAs of the library (though none was detected during the optimisation process) but more likely due to the aforementioned variability of this assay. It was, therefore important to validate as many preliminary hits as possible by retesting in the same assay, both to verify the efficacy of the screen and to prevent premature exclusion of any potential leads.

6.3.2 Screen results and hit validation

Consideration of the preliminary hit lists (Z-score <1.5) for both the 10nM and 6nM screens (Fig 4.4) revealed a number of known DSB repair proteins

identified towards the top of both hit lists. In both the 6nM and 10nM screens, the key HR mediator BRCA2 together with its interacting protein DSS1 were identified with Z-scores of <-2 . Although each screen identifies a number of DSB repair proteins at the top of the hit list, there were notable exceptions, including RAD51. Extension of the Z-score cutoff from under -2 to under -1.5 extends the list of DSB repair proteins retrieved by the screen, with BRCA1, BRCA2 interaction partner PALB2 and the ssDNA binding RPA1 all identified. These observations both lend confidence to the screening strategy as being capable of identifying HR proteins, whilst demonstrating that such a screen cannot be expected to identify all proteins required for HR by this (or perhaps any) screening method. The recently published genome-wide RNAi DR-GFP screen [182] provides a convenient dataset for comparison. Their work also identifies very few known DSB repair proteins as hits with only BRCA1, DSS1, RAD51, BARD1, RAD51AP1, RNF168, EME1 amongst their list of 228 hits [182]. The paucity of HR proteins identified in this study is in line with my own observations and I believe reflects some of the limitations of the DR-GFP assay with respect to high-throughput screening (discussed earlier in this Chapter). Furthermore the lack of correlation with my hit-list (Fig 4.4) may be in part accounted for by the different approach used by the authors of the genome-wide DR-GFP screen [182], specifically their simultaneous use of RNAi at the same time as DSB induction in comparison to my use of RNAi in advance of DSB induction as discussed earlier.

The preliminary hit list was initially defined as all those siRNAs taken from the two siRNA screens I performed that had a Z-score of <-1.5 , plus BRD1 (Z-score -1.3) as a poorly characterised bromodomain-containing protein of potential interest. The majority of these recapitulated their originally observed phenotypes upon retesting in the DR-GFP assay (Fig 4.5 (a)), underlining the reliability of the initial hit selection process. One exception was c9orf102, which gave a decrease in the DR-GFP activity but with high replicate variability, making it statistically insignificant. Subsequent testing of c9orf102 in the deconvolution assays verified the initial screening result.

Having validated the majority of the preliminary hits (Fig 4.5), the hit list was further reduced by removing siRNAs targeting all known DSB repair proteins and those for which a mechanism of HR involvement was difficult to conceive - such as COPB2 (Golgi protein trafficking) and NUP205 (nucleoporin). Each siRNA of this curated hit list was then tested for its ability to sensitise cells to DNA damage (in this case induced via MMC treatment, leading to ICL formation) [Fig 4.5]. Most members of the curated list demonstrated some sensitising effect, bolstering confidence in the screens. It is important to appreciate that such a sensitisation assay with a cell viability endpoint, conducted at a single concentration of MMC is not likely to be as sensitive as a dose response clonogenic survival assay. This technical limitation, necessitated by the requirement to test a large number of siRNAs may well account for the fact that JMJD1B knockdown showed no effect in the MMC sensitisation assay but later demonstrated sensitisation to both

CPT and MMC in clonogenic survival assays (Fig 4.14). With respect to MMC sensitisation assays, it is worth bearing in mind that although RAD51 knockdown and therefore HR inhibition sensitises cells to MMC treatment (Fig 4.14), MMC induced damage (ICLs) may also be directly repaired via non-HR mechanisms, specifically the FA repair pathway (Fig 1.6). Thus a sensitisation to MMC may not in itself demonstrate that HR is inhibited. It is for this reason that sensitisation assays with multiple DNA damaging agents were conducted which, together with the data gathered from the DR-GFP and foci-formation assays could be used to infer whether or not a particular siRNA was likely to be mediating the observed effects via HR inhibition.

6.3.3 siRNA hit deconvolution

The siRNA hit validation process allowed selection of hits for further investigation. Led by promising results in the initial screens, the DR-GFP validation and the MMC sensitisation experiments (in which c9orf102 emerged as the top hit), a list of siRNAs for follow up work was defined: BRD1, c9orf102, CHD8, JMJD1B, SUV3, TET3 (Figs 4.7 and 4.8).

These selected hits were all subjected to deconvolution multiple deconvolution assays. Deconvolution in the the context discussed within this thesis represents the individual testing of the four constituent siRNAs that are combined to create the pooled siRNA reagent that was initially used for the siRNA screen. A successful deconvolution experiment is defined as at least two of the four siRNAs of a pool mediating the same effect in a given assay.

All selected hits, except CHD8, were successfully validated in the DR-GFP assay (at least two of the constituent siRNAs of the pool exhibiting the desired phenotype). The CHD8 deconvolution did show a modest DR-GFP inhibitory effect from two of the siRNAs, which are likely to combine to give the phenotype of the pooled siRNA as witnessed in the initial screen and subsequent DR-GFP validation experiment. This modest effect was not deemed sufficient to warrant further work.

The qPCR deconvolution data showed an overwhelming efficacy (high levels of transcript knockdown) of nearly all the siRNA reagents highlighting an inconsistency between the DR-GFP and qPCR deconvolution data. A possible explanation is that the constituent siRNAs of each pool have differing stabilities once inside the cell. This may account for why nearly all siRNAs tested gave effective transcript knockdown 24 hours after transfection but didn't all yield an inhibitory effect on the DR-GFP assay whose endpoint is 96 hours after siRNA transfection. Throughout the optimisation process there was no evidence to suggest that effective protein knockdown was not maintained until 96 hours after siRNA transfection (Figs 3.4 and 3.6), but this possibility cannot be discounted for the range of siRNAs looked at in this study. Further appreciation of the phenotypic variability between individual siRNAs of a pool may be gained when one considers that the algorithms used to generate siRNAs give a probability of 80-90% for each siRNA reducing target transcript levels by 70% [106]. This limitation may be coupled to different phenotypes emerging from a range of transcript knockdown levels

as a result of inter-pool variability with regards to potency [106].

The success rate of the primary validation (retest in DR-GFP assay), complementary assay (MMC sensitisation) and deconvolution experiments is consistent with observations from other large scale siRNA screens in the DNA damage field. For example, the genome-wide DR-GFP screen of Slabicki *et al* [182] identified 228 hits for potential HR inhibitory effects; of these only 45 (20%) were validated with independent siRNAs. Similar results are recorded for genome-wide DNA crosslinker and IR sensitisation screens [183, 85] in which 22% and 40% of hits validate in a complementary assay. Consideration of my deconvolution data (5/6 selected hits validated successfully in DR-GFP and qPCR assays, 3/6 in foci-formation assays) in light of published work is favourable with deconvolution success rates in different screens in the range of 50-70% [182, 161]. It can be difficult to compare screens from different laboratories due to the diversity of reagents, assays, data analysis methods, statistical cut-offs and follow-up procedures used but I believe the quality of the screening and follow-up data shown here are at least in line with other examples in the literature.

6.3.4 Hit characterisation: BRD1, JMJD1B, TET3

Successful deconvolution experiments for BRD1, JMJD1B and TET3 siRNAs (as well as c9orf102 and SUV3 to be addressed separately, below) verified the relevance of my screening approach, in each case atleast two of the four siRNAs against each target inhibited the DR-GFP assay (Fig 4.7) as well

as producing significant transcript knockdown as measured by qPCR (Fig 4.8). Further characterisation in foci-formation assays (Fig 4.9) was able to show that the pattern of the deconvolution results (i.e. consistent effects mediated by the same siRNAs in different assays) in the DR-GFP and RAD51 foci readouts was comparable for BRD1, JMJD1B and TET3, suggesting that target knockdown in all cases may inhibit HR via impairing RAD51 nucleoprotein filament formation - a plausible HR inhibitory mechanism.

The trend across the BRD1, JMJD1B and TET3 siRNA deconvolution experiments was broadly maintained when considering the 53BP1 and RPA foci formation data: BRD1 knockdown causes a modest reduction of 53BP1 and RPA foci formation with a similar pattern to that seen in the RAD51 deconvolution experiment (Fig 4.9). Further work is necessary to define whether the subtle effects of BRD1 knockdown on 53BP1, RPA and RAD51 foci formation are biologically relevant. Verification of protein knockdown in this case would be particularly helpful, although at the time of writing we have not acquired a BRD1 antibody. The data so far suggest that BRD1 knockdown impairs HR via an inhibitory effect on both 53BP1, RPA and RAD51 foci formation.

JMJD1B knockdown inhibited formation of all the foci markers used and in particular RAD51 (Fig 4.9). As with BRD1, this effect on 53BP1-, RPA- and RAD51 foci-formation suggests a role for JMJD1B at the very beginning and possibly throughout the damage recognition process, before HR has been initiated. Contrary to expectations, the JMJD1B siRNA deconvolution for

transcript and protein knockdown contrasts with the experimental data from the DR-GFP and foci formation assays (Figs 4.8, 4.9, 4.10, 4.11, 4.12 and 4.13). JMJD1B siRNA 1 does not show strong transcript and protein knockdown but demonstrates a strong inhibition of RAD51 foci-formation. These discrepancies between protein knockdown and HR inhibition cannot easily be accounted for. One possibility is that each siRNA of the pool differs in its stability resulting in effective protein knockdown at different timepoints for each reagent. Further timecourse analysis of JMJD1B protein knockdown following siRNA transfection would allow an assessment of this hypothesis.

The TET3 deconvolution data indicate that inhibition of the DR-GFP assay by TET3 siRNA is consistent with an inhibitory effect of TET3 knockdown on RAD51 foci formation. The pattern of DR-GFP and RAD51 foci-formation inhibition across the four TET3 siRNAs is almost identical (Figs 4.7 and 4.9). The TET3 siRNAs display a limited effect on 53BP1 and RPA foci formation (Fig 4.9). In contrast to both BRD1 and JMJD1B, the potential role of TET3 in HR appears to be restricted solely to events downstream of resection, during the formation of the RAD51 nucleoprotein filament. Unfortunately, as with BRD1, I currently do not have access to a validated TET3 antibody.

With promising data acquired from the DR-GFP and foci formation deconvolution experiments for BRD1, JMJD1B and TET3, and with the pooled siRNA reagent validated in each case, I determined whether knockdown of each target sensitised cells to DNA damaging treatments (specifically CPT

and MMC). In line with expectations, both BRD1 and JMJD1B knockdown demonstrated such an effect whilst, in contrast to the data collected from the DR-GFP and RAD51 foci-formation assays, TET3 knockdown did not. In the absence of a suitable antibody, it is difficult to assess whether the lack of sensitising effect in the case of TET3 is a result of inconsistent protein knockdown by TET3 siRNA across the different experiments.

Some of the inconsistencies throughout the deconvolution process, for example as displayed by JMJD1B siRNA 1 (see above) may be accounted for by off-target effects of individual siRNAs. This uncertainty remains for all of the hits I have worked with to this point, including those discussed below, and can only be addressed with successful rescue experiments. Rescue experiments will entail the restoration of function in the assay of choice using cells in which the endogenous transcript has been knocked down. This is achieved by the expression of an siRNA-resistant construct coding for the protein under study. This work requires both a suitable expression vector (ideally siRNA-resistant) or siRNAs for the relevant target that bind to one of the untranslated regions (UTRs) of the transcript. In addition, for an optimal rescue experiment, a cell line stably expressing the construct of interest is highly desirable. Generation of stably expressing cell lines for those hits of immediate interest (SUV3, BRD1 and c9orf102) is currently underway in our laboratory.

BRD1, JMJD1B, chromatin remodelling and HR

Taken as a whole, the characterisation experiments for BRD1, JMJD1B and TET3 support the hypothesis that both BRD1 and JMJD1B are required for effective HR. Any potential role for TET3 is more difficult to support. It is also possible to speculate at which points in the damage recognition and HR pathways these proteins act based upon the foci formation data - though further work is required to solidify such predictions.

The nature of BRD1 and JMJD1B as a bromodomain-containing protein [138] and a histone lysine demethylase [3], respectively, suggests an indirect mechanism for involvement in HR in both cases: BRD1, so named for its histone acetyl lysine binding bromodomain, also contains 2 PHD zinc fingers characteristic of a chromatin regulator and has recently been described as part of a histone acetyl transferase (HAT) complex in partnership with the HBO1 HAT [3]. The HBO1-BRD1 complex is responsible for the majority of histone 3 lysine 14 acetylation in the cell [138]. Additional recent work has described the role of this particular histone modification in activating DNA-PK under hypoxic conditions [17]. How these observations might relate to a role for BRD1 in HR is not clear, but investigation of the relationship between BRD1-HBO1 mediated histone acetylation and the DNA damage response is certainly warranted in light of the data presented here.

JMJD1B is thought to be a histone H3, lysine 9 demethylase [3], although observations of this activity have not been published. Histone H3, lysine 9 tri-methyl demethylation activity has been associated with transcriptional

derepression and chromatin remodelling [3]. This activity is likely to be of biological and perhaps medical significance since deletions in the genomic region coding for JMJD1B have been associated with myeloid malignancies and it has been speculated that this region may contain a tumour suppressor gene [79, 124]. Whether the observed HR inhibition resulting from JMJD1B knockdown shown by my work is linked to JMJD1B's role in chromatin remodelling and how such a connection may contribute to a prospective function of JMJD1B as a tumour suppressor is the subject of work being actively pursued in our laboratory.

Key to revealing the possible relationships between the histone modifications mediated by BRD1 and JMJD1B is the robust demonstration of their HR promoting activity and whether the histone modifications they catalyse are required for this process. These avenues of research have been opened up by the screening and validation work described herein.

6.3.5 Hit characterisation: c9orf102/RAD26L

Early in the hit validation process, my attention focused on the uncharacterised protein c9orf102 (Figs 4.16 - 4.20). Sequence analysis identifies this predicted protein as a relative of the yeast nucleotide excision repair helicase Rad26 and its human counterpart ERCC6/Cockayne syndrome B (CSB) and defines c9orf102 or RAD26L as a SF2 family helicase/translocase. These predictions have yet to be validated experimentally, for example by *in vitro* helicase activity assays.

Deconvolution of the four c9orf102 siRNAs in the DR-GFP and qPCR assays was successful (two of the four c9orf102 siRNAs exhibited both DR-GFP inhibitory activity as well as significant transcript knockdown) and I established that c9orf102 knockdown inhibits both RPA and RAD51 foci formation following DNA damage induction. The deconvolution experiments demonstrated that c9orf102 siRNAs 1 and 4 had consistent inhibitory effects across all assays and probably drove the phenotype induced by use of the pooled reagent. At this stage it was noted that c9orf102 1 and the pooled siRNA reagent were both toxic to cells, accordingly the c9orf102 4 siRNA was used to demonstrate a marginal sensitisation of c9orf102 knockdown cells to CPT and MMC, in line with expectations from the deconvolution experiments.

With a view to performing rescue experiments to verify my findings thus far, siRNA directed against the 3'-UTR of c9orf102 (c9orf102 13) tested for its HR inhibitory effects in the DR-GFP, RAD51 foci formation and clonogenic survival assays conducted with MMC, CPT and IR. In all cases, the expected HR inhibitory effects were confirmed, both increasing confidence in my hypothesis that c9orf102 is required for efficient HR and facilitating rescue experiments (Fig 4.17).

The rescue experiments (ectopic expression of c9orf102 in cells treated with c9orf102 13 siRNA) did not restore function in either the DR-GFP or RAD51 foci formation assays, the reasons for this lack of rescue may be technical, arise from the possibility that the c9orf102 expression construct

was not the full sequence (see below and Fig 4.20) or serve as an indication that the previously observed effects of c9orf102 knockdown are due to off-target effects. Possible technical explanations may arise from the difficulties of restoring function in cells that have undergone a transient siRNA transfection to knockdown the target protein, followed by a transient transfection of the c9orf102 rescue vector. Using such a protocol, it is impossible to guarantee that cells successfully transfected with siRNA will all undergo rescue vector transfection. To address these problems, it is necessary to generate either a cell line with a stable c9orf102 knockdown or conversely cell line with stable, siRNA-resistant, c9orf102 expression. In advance of performing these time-consuming steps, I sought clarification of the nature of the c9orf102 transcript by Northern blot (with the assistance of Dr C. Bachrati, Helleday laboratory). In striking contrast to the expected length of the predicted transcript (4666 bases), the endogenous transcript detected using a full-length (as predicted in the PubMed gene database) c9orf102 cDNA was found to be in the region of 7500-8000 bases. This discovery posed several questions relating to the true sequence of c9orf102. It is possible that the full length protein-coding c9orf102 gene is longer than predicted - such an eventuality could offer an explanation for the failed rescue experiments due to ectopic expression of a truncated construct. Reassuringly, the c9orf102 13 siRNA against the predicted 3'-UTR of c9orf102 efficiently knocks down levels of the detected c9orf102 transcript, suggesting that knockdown of this uncharacterised transcript is indeed responsible for the HR-inhibitory effect

shown by my data (Fig 4.17). Verification of c9orf102 protein knockdown has proved elusive, with only poor-quality commercial antibodies currently available.

My work relating to c9orf102 leaves a number of important questions unanswered, however, until the exact sequence of the c9orf102 mRNA and protein can be experimentally determined, it is difficult to address these with any certainty. A crucial initiating step in this work will be the cloning sequencing of the full length c9orf102 transcript.

6.3.6 Hit characterisation: SUV3

Perhaps the most promising data arising from the siRNA screen relates to the SUV3 helicase (Figs 4.21 and 4.22). This multifunctional helicase with dsRNA, dsDNA and RNA:DNA hybrid unwinding activities [180] was originally characterised in humans by virtue of its homology to Suv3 in yeast and is conserved from *Rhodobacter* to eukaryotes [40]. Early work demonstrated a distinct mitochondrial role for SUV3 in regulating RNA turnover as part of the mitochondrial degradosome [136, 97] and further studies have illuminated an additional, nuclear role for SUV3 with biochemical interactions identified with both BLM and WRN helicases [162] as well as immunoprecipitation experiments showing interactions in the nucleus with RPA and the flap endonuclease FEN1 [210]. This recent study has expanded the biochemical understanding of SUV3, revealing RPA-mediated inhibition of SUV3 helicase activity, whilst SUV3 itself was found to stimulate FEN1 activity [210].

What role these interactions play within the cell in the context of HR remains to be seen.

In contrast to the other hits selected during my work, SUV3 is well already well characterised allowing a more informed consideration of my data. SUV3 knockdown leads to marked inhibition of the DR-GFP and RAD51 foci formation assays and to a lesser extent RPA foci formation (Figs 4.7 and 4.21). These discoveries are in agreement with a proposed nuclear role for SUV3 and the recently characterised SUV3 interaction with RPA [210]. Whether this interaction explains the effect of SUV3 knockdown on RPA foci formation is an intriguing possibility.

Fortuitously, a SUV3 antibody was generously made available to me by Prof. P. Stepien [136], greatly aiding validation of the SUV3 siRNAs used throughout my work. In agreement with the pattern of deconvolution data for the DR-GFP and RAD51 foci assays, I found that all SUV3 siRNAs except siRNA 4 efficiently knocked down SUV3 protein levels (Fig 4.22). At the same time as validating SUV3 knockdown, I noticed that RAD51 protein expression levels were greatly reduced in tandem with SUV3 levels by the validated SUV3 siRNAs (Fig 4.22). This serendipitous discovery may give an insight into how HR inhibition is mediated by SUV3 knockdown and accounts for the sensitisation of SUV3 deficient cells to a range of DNA damaging agents (MMC, CPT and IR), although it does not explain its effect on RPA foci formation. Reassuringly the pattern of SUV3 knockdown across the different SUV3 siRNAs matched that of the RAD51 knockdown

i.e. those siRNAs that produced strong SUV3 knockdown had the same effect upon RAD51 protein levels.

With the realisation that SUV3 knockdown may inhibit HR by modulating RAD51 expression levels, it will be important to dismiss non-SUV3 specific mechanisms for this phenomenon. One obvious candidate is apoptosis, which has been described to be induced by SUV3 knockdown in HeLa cells [200]. None of the siRNAs used during the course of my work are toxic and cleaved RAD51 that may be expected as a result of caspase 3 activity in apoptotic cells [82] is not detected in my Western blots (Fig 4.22). Further demonstration that RAD51 levels are not reduced via an apoptotic mechanism and that apoptosis is not induced following SUV3 knockdown will be required to fully dismiss these concerns. Analysis of RAD51 transcript levels following SUV3 knockdown will also aid the understanding of how the proposed SUV3 regulation of RAD51 levels is achieved.

Following from the results of the c9orf102 rescue experiments, in which no rescue of function in both the DR-GFP and RAD51 foci-formation assays was observed, it was decided that cell lines stably expressing SUV3 should be generated to avoid technical problems relating to transient ectopic expression systems. These cell lines are currently being prepared in our laboratory and will allow verification that the siRNAs used in my work are on-target.

6.3.7 siRNA screen - future directions

The follow-up work derived from the initial siRNA screen datasets has uncovered a number of promising leads for potentially novel HR proteins (BRD1, c9orf102 and SUV3), satisfying the initial aim of this arm of the project. In all cases, two major steps are required to robustly confirm the assumption that the validated hits (BRD1, JMJD1B, c9orf102 and SUV3) all play a role in promoting and/or enabling HR. Firstly, verification of protein knockdown using specific antibodies against each target (already achieved for JMJD1B and SUV3) and secondly, successful rescue of the HR-inhibitory phenotypes described in each case. Only after these experiments are successfully completed can further characterisation work be pursued and it remains possible, though unlikely, that one or more of the hits has been identified purely due to off-target siRNA effects.

Initial work, pending the satisfaction of the two conditions listed above, will focus on whether or not any of these prospective HR proteins localise to sites of DNA damage. These data will facilitate clarification of whether a hit from my screen exerts its effect on repair directly as part of the repair or damage recognition machinery proximal to the damaged DNA, or alternatively indirectly via global changes. Regulation of DNA accessibility via the modulation of chromatin remodelling activity is a possible indirect mechanism for BRD1, JMJD1B and even c9orf102 and SUV3. In the cases of BRD1 and JMJD1B, in particular, it will be important to characterise whether the inhibitory effects on HR following BRD1 and JMJD1B knock-

down so far described are specific to chromatin remodelling. In light of the results anticipated from such experiments more detailed studies may then be designed to address the mechanism of any novel HR protein identified.

Although BRD1, JMJD1B and c9orf102 all represent attractive potential HR proteins, I believe the most immediately promising candidate for further work is SUV3. This belief is founded on the persuasive experimental data that I have collected combined with observations from other groups asserting a nuclear function for this helicase, perhaps in partnership with RPA. Further work in our laboratory will initially be directed towards firmly demonstrating that SUV3 is a novel HR protein, whilst a more fundamental understanding of the basic biology of BRD1, JMJD1B and especially c9orf102 needs to be developed by our group and others before defining any functions in HR.

6.4 Small molecule screen

Although two small molecule screens were performed, the kinase inhibitor screen by hand and the NCI screen with assistance from the robotic handling station, these will be considered together as a number of issues arose during the course of my work with relevance to them both.

Assay performance

The most immediately obvious drawback with both screens was the poor replicate data quality (Figs 5.1 and 5.5), which was more pronounced in the kinase inhibitor screen and was a particular problem for those compounds

that produced an increase in the relative percentage of GFP positive cells in the DR-GFP assay. There was some improvement in the replicate data quality in the NCI screen as compared to the kinase inhibitor screen and I believe this may be explained firstly by the use of early passage UGFP1 cells together with the use of the robotic handling station for conducting the drug treatment steps of the NCI screening protocol.

Of concern in the NCI screen, in particular, was the variable effect of the positive control (0.4, 0.6 μ M PCI). In most cases, treatment with PCI reduced the percentage of GFP positive cells relative to the DMSO and blank controls, but this effect was not universal across all plates of the screen. Whilst it is possible in such a large scale experiment that pipetting errors may occur, I have also observed over the course of my work that the effect of PCI treatment on HR activity, as measured by the DR-GFP, RAD51 foci formation and DNA damaging drug sensitisation assays is variable and not as consistent as one could hope. These observations may be rationalised when one considers the nature of this drug: PCI is a broad spectrum HDAC inhibitor and as such would be expected to have diverse effects upon chromatin dynamics and DNA accessibility. Whilst these effects are most likely to be responsible for the PCI-dependent inhibition of HR via modulation of RAD51 expression levels, it is difficult to predict what spectrum of other proteins and pathways might be disrupted by non-specific HDAC inhibition such as that mediated by PCI. Aside from its effect on RAD51 expression levels that directed my desire to use this compound as a positive control, PCI

treatment has also been shown to downregulate expression of NF- κ B, antioxidant and proteasome genes as well as inducing caspase and reactive oxygen species-dependent apoptosis [15]. The competition between these known and the highly probable, unknown effects of PCI treatment may drive the effects seen in my assays and I believe may account for the variable results seen. A better understanding of the concentrations of PCI that mediate each of the different known effects would enable a more reliable assessment of whether or not this compound is a suitable positive control for HR inhibition. The problems experienced with PCI serve to highlight the importance of identifying specific HR inhibitors and suggest that use of an alternative positive control could have been pursued. However, our lab had acquired a large quantity of PCI via a materials transfer agreement with its manufacturer (Pharmacyclics) prior to the beginning of my project. Due to the large amounts of compound required for high-throughput screening, I elected to use these pre-existing compound stocks.

6.4.1 Screen results and hit validation

With the screens completed and with an awareness of the likelihood of false positives, hit validation was of great importance (Figs 5.2, 5.8 and 5.9). In the case of the kinase inhibitor screen, it was reassuring to identify the known HR inhibitors (the CHK1 and CHK2 inhibitor AZD7762 [141] and the HSP90 inhibitor Geldanamycin [154]) amongst the preliminary hit compounds. Furthermore, 15/17 preliminary hit compounds (the known HR inhibitors were

not followed up) gave favourable dose responses in the DR-GFP i.e. a clear dose response and an inhibition of the DR-GFP assay in excess of their toxicity (Fig 5.2). In contrast and perhaps reflecting both the nature of the compound library and scale of the screen, the retesting of the 134 preliminary hits defined from the NCI screen in the DR-GFP assay revealed only around a third of hits that maintained their effects. Of these, the majority (17/22) displayed a favourable dose response in the DR-GFP assay.

The outcome of the validation process in both screens revealed that the screens were effective at identifying numerous compounds with significant and repeatable inhibitory activity of the DR-GFP assay, but which failed to universally validate in the subsequent characterisation assays. It is highly likely that this result stems from a lack of specificity in the DR-GFP assay and those compounds identified as DR-GFP assay inhibitors but with no effect in alternate assays have an inhibitory effect on the production of GFP, possible at numerous steps in the transcription, translation and protein maturation process. The aforementioned inclusion of a transfection control for the I-SceI transfection step of the assay (e.g. DsRed tagged I-SceI) would have potentially reduced the identification of such false positives.

6.4.2 Hit characterisation

It was now necessary to screen all the validated hit compounds from both the kinase inhibitor and NCI compound screens in complementary assays to further establish the veracity of any HR inhibitory effect and to aid with

characterisation of the mechanism of action of the hit compounds (Figs 5.3, 5.5 and 5.10 - 5.12). In the case of both sets of preliminary hits (kinase inhibitor and NCI libraries), compounds were identified that had no significant effect upon any of the foci formation assays that was in excess of their toxicity, yet sensitised cells to CPT treatment. It is reasonable to assert that such compounds that (at non-toxic levels) induce inhibition of the DR-GFP assay and sensitise cells to DNA damage, have an inhibitory effect on HR. A more difficult assertion to make is that this effect is specific to HR. Furthermore it is difficult to predict what the target of inhibition may be, even in the case of the kinase inhibitors, all of which have relatively broad inhibitory profiles.

Follow up work performed on the NCI hits also identified two compounds (28 and 96) that have pronounced inhibitory effects on both RPA and RAD51 foci formation at non-toxic concentrations, whilst leaving 53BP1 foci formation unaffected. Though still far from defining a target for these compounds, these results lend confidence to the hypothesis that these are HR inhibitors and allow speculation that they mediate an inhibitory effect downstream of DNA damage recognition and upstream of either resection or RPA loading. The generation of such an hypothesis is of great value in guiding future characterisation experiments of these particular hits.

Based upon the promising data collected for NCI hits 28 and 96 with respect to the DR-GFP and foci formation assays, it was expected that they would sensitise cells to DNA damage treatment. The CPT sensitisation assay did not confirm this prediction, though it must be noted once more

that such an assay is not as sensitive as lower throughput (e.g. clonogenic survival assays) that may now be used, with multiple DNA damaging agents, to further characterise these compounds.

The lack of a high degree of cross-validation between the DR-GFP and DNA damaging agent (CPT) sensitisation assays may also reflect the complex nature of CPT induced damage repair (discussed earlier in this Chapter), testing of the DR-GFP assay validated hits in sensitisation assays with a number of different DNA damaging agents (e.g. the radiomimetic bleomycin) may have circumvented this problem, though such an approach is prohibitive for a large number of compounds being used at multiple concentrations.

Target identification

Should the expected DNA damage sensitising effect be successfully demonstrated for NCI hits 28 and 96, confidence in their roles as HR inhibitors would be sufficient to attempt the identification of their targets. Such an endeavour is not to be taken lightly. One approach that has been successfully applied for target identification is the chemical immobilisation of the hit compound onto an affinity tag or solid matrix to allow the application of affinity pull-downs or chromatography, respectively, followed by mass-spectrometry to identify proteins that bind to the hit of interest [119]. This technique requires the generation of a reactive chemical linker to attach the compound of interest to the tag/matrix in such a position so as not to mitigate the binding activity of the compound and also relies on a high affinity interaction

between ligand and target. Such work would be a significant undertaking, however, target identification is an inevitable hurdle imposed with cell-based screens such as those conducted here, as was acknowledged from the outset.

6.5 Conclusions

Development of the DR-GFP assay for high-throughput screening

One implicit aim of the project was to develop a high-throughput compatible HR activity assay that is compatible with both siRNA and small molecule work. Though not without its limitations, the DR-GFP assay was developed successfully for this purpose. This conclusion may be drawn when considering the results obtained for both the siRNA and small molecule screens. Whether the DR-GFP assay is the most appropriate tool for such work remains debatable. The most viable alternative cellular HR assay compatible with high-throughput would probably be a foci formation assay, though the resultant data quality and ability to detect genuine HR proteins is still far from ideal (Dr C. Lundin, Helleday laboratory, unpublished work). In conclusion, I believe that the success of such a cell based screen lies in the appropriate application of complementary assays that can both confirm the initial activity displayed in the primary screen, whilst informing upon any possible mechanisms of action to guide the direction of future work.

Identification of novel HR proteins

The siRNA screen and resultant follow up work has served to identify four candidate novel HR proteins. There remains a number of experiments in each case that are required to fully satisfy the hypothesis that BRD1, c9orf102, JMJD1B and SUV3 promote or enable HR but all my work so far is in line with this prediction and has firmly established these hits as leading candidates and laid the foundation for continuing work in our laboratory.

Identification of novel HR inhibitors

From the outset, this element of the project was likely to be the most challenging and this proved to be the case. The lack of target knowledge inherent to such work makes characterisation of any hits a particular problem. Nevertheless, a number of potential HR inhibitors have been identified through the screening process and provide the confidence required to demand further follow up work to establish how HR inhibition is achieved and how specific this observed effect is in the case of each hit.

Perspectives

I believe that my work has opened up multiple avenues of research for my laboratory. The identification through high-throughput siRNA screening of potential novel HR proteins in addition to those recently identified and characterised in similar studies [85, 182, 161, 121] demonstrates that this is still a fertile area of research where much is left to be discovered. The presence,

amongst my hits, of numerous siRNAs targeting chromatin remodelling proteins also underpins the growing realisation that the chromatin context within which DNA repair takes place is of great importance to the maintenance of genomic stability [58, 83, 127, 103, 54, 38]. Finally, my identification of candidate HR inhibitors poses the daunting but fascinating question of their biological target(s) which, if answered, will enable the pursuit of further research into the clinical relevance of HR inhibition as a cancer treatment.

Appendix A

Appendix

A.1 siRNA library

A 'Flexiplate' siRNA library was purchased from Qiagen and constitutes siRNAs against all known and putative DNA repair genes and a number of chromatin remodelling genes. Each well contained a pool of four individual siRNAs against a common target. There were 579 targets in total.

For deconvolution experiments, each of the 4 siRNAs of the constituent pool (the identical sequences to those used in the screen) were ordered separately from Qiagen.

The siRNAs used as controls are listed below in Table A.1. In all cases for control siRNAs, the individual siRNAs were pooled. In the cases of 53BP1 and RPA, only two siRNA sequences were pooled; both of these had been validated by the vendor (Qiagen - www.qiagen.com).

Table A.1: Target sequences of siRNAs used herein. All siRNAs were purchased from Qiagen, with the exception of XRCC3 (Dharmacon).

name	sequence
MLH1 6	GTGGCTCATGTTACTATTACA
MLH1 7	AACCATCGTCTGGTAGAATCA
MLH1 4	CAGTGGATTGATTATAAATAA
MLH1 3	CACCAGATGGTTCGTACAGAT
RAD51 7	AAGGGAATTAGTGAAGCCAAA
RAD51 1	CAGGATAAAGCTTCCGGGAAA
RAD51 8	CACGGTTAGAGCAGTGTGGCA
RAD51 6	CACTTCTAAATTAATGGTAAA
RPA1 6	CCGTGTGACGATCCCATGTTA
RPA1 5	CAGGAATTATGTCGTAAGTCA
WRN 6	CGGATTGTATACGTAACTCCA
WRN 9	TCCGCTGTAGCAATTGGAGTA
WRN 8	CTACGTGACTTTGATATCAAA
WRN 7	CCACGGAGGGTTTCTATCTTA
XRCC3 1	GGACCUGAAUCCCAGAAUU
XRCC3 2	GCUGAGAACGGCCUCCUUA
XRCC3 3	GGACCAGACUUGAAGAGAC
XRCC3 4	UGAAAUCGGUAAAGGAGGU

53BP1 8	CAGGACAGTCTTTCCACGAAT
53BP1 7	TTGTTTGACCAGAGCAATAAA
CHD8 9	CCCAAGGATCGTGTCTGATA
CHD8 8	CAGAACATACCTCGAGTCTTA
CHD8 7	CAAGATAGTGTTACAGGGCAA
CHD8 5	CCGGATCCCTGTCATCAATAA
BRD1 4	ACGGTTAGAAGCTCAAGGGTA
BRD1 3	AAGACGGGATGTAAATTTACA
BRD1 2	TCCTGTCTGCTTAAGAACTAA
BRD1 1	CTGACCTACGCTCAAGCTCAA
JMJD1B 8	TGGAGCGGTACCGGAAGTTTA
JMJD1B 7	CTGGGATGGTTTCGAGATCAT
JMJD1B 6	ATGGATAATTCAACGCCTCAA
JMJD1B 5	ACCGAAGGGTCTAGCCTTCGA
TET3 4	CCGCATCTCGCTGGTCTTCTA
TET3 3	TTCCGTGATCTTAATATTTAT
TET3 2	CCGGATCGAGAAGGTCATCTA
TET3 1	CCGGCTCTATGAAACCTTCAA
c9orf102 11	ACCAAGTTGCTTGACGTGCTA
c9orf102 9	CTGGCCGAAAGGCCATGCAAA
c9orf102 8	TAGGTATTTGAGAGACTACCA
c9orf102 7	CCGCGCGGAAACCTCAGGCAA

A.2 qPCR primers

Primers used for qPCR were generated using PerlPrimer software and are listed in Table A.2.

Table A.2: qPCR primers.

name	direction	sequence
BRD1	forward	GGAGGCCTCTGAATATTTACG
BRD1	reverse	GCAATCCTATTTAACCTCTGCG
c9orf102	forward	CATTGACAGAGCATATAGGATTGG
c9orf102	reverse	CCTTCGTAAGACAAGACCCT
CHD8	forward	TCATTCAGGTCTATCTATCCCT
CHD8	reverse	CTCAGGTAGTATAGCATTCGT
JMJD1B	forward	ACAACCTCTCTTCAACATCCAC
JMJD1B	reverse	CTTCTTCATCACCCATCTCTTC
SUV3	forward	GGGACTTAATTTGAGCATAAGG
SUV3	reverse	CTTGAGAGGTTGTGATTGGT
TET3	forward	CGATTGCGTCGAACAAATAG
TET3	reverse	CTTTCCCCTTCTCTCCATACC

Bibliography

- [1] S. Adimoolam, M. Sirisawad, J. Chen, P. Thiemann, J. M. Ford, and J. J. Buggy. HDAC inhibitor PCI-24781 decreases RAD51 expression and inhibits homologous recombination. *Proc Natl Acad Sci U S A*, 104(49):19482–7, 2007.
- [2] M. Aggarwal, J. A. Sommers, R. H. Shoemaker, and R. M. Brosh, Jr. Inhibition of helicase activity by a small molecule impairs Werner syndrome helicase (WRN) function in the cellular response to DNA damage or replication stress. *Proc Natl Acad Sci U S A*, 108(4):1525–30, 2011.
- [3] K. Agger, J. Christensen, P. A. C. Cloos, and K. Helin. The emerging functions of histone demethylases. *Curr Opin Genet Dev*, 18(2):159–68, 2008.
- [4] A. Ahmad, A. R. Robinson, A. Duensing, E. van Drunen, H. B. Beverloo, D. B. Weisberg, P. Hasty, J. H. Hoeijmakers, and L. J. Niedernhofer. ERCC1-XPF endonuclease facilitates DNA double-strand break repair. *Mol Cell Biol*, 28(16):5082–92, 2008.

- [5] A. Z. Al-Minawi, N. Saleh-Gohari, and T. Helleday. The ERCC1/XPF endonuclease is required for efficient single-strand annealing and gene conversion in mammalian cells. *Nucleic Acids Res*, 36(1):1–9, 2008.
- [6] A. M. Ali, T. R. Singh, and A. R. Meetei. FANCM-FAAP24 and FANCI: FA proteins that metabolize DNA. *Mutat Res*, 668(1-2):20–6, 2009.
- [7] A. D. Auerbach, S. R. Wolman, and R. S. Chaganti. A spontaneous clone of Fanconi anemia fibroblasts with chromosome abnormalities and increased growth potential. *Cytogenet Cell Genet*, 28(4):265–70, 1980.
- [8] J. Bartek, J. Bartkova, and J. Lukas. DNA damage signalling guards against activated oncogenes and tumour progression. *Oncogene*, 26(56):7773–9, 2007.
- [9] J. Bartkova, Z. Horejsi, K. Koed, A. Kršmer, F. Tort, K. Zieger, P. Guldberg, M. Sehested, J. M. Nesland, C. Lukas, T. Ørntoft, J. Lukas, and J. Bartek. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature*, 434(7035):864–70, 2005.
- [10] J. Bartkova, N. Rezaei, M. Lontos, P. Karakaidos, D. Kletsas, N. Isaeva, L. V. Vassiliou, E. Kolettas, K. Niforou, V. C. Zoumpourlis, M. Takaoka, H. Nakagawa, F. Tort, K. Fugger, F. Johansson, M. Sehested, C. L. Andersen, L. Dyrskjot, T. Ørntoft, J. Lukas, C. Kit-

- tas, T. Helleday, T. D. Halazonetis, J. Bartek, and V. G. Gorgoulis. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature*, 444(7119):633–7, 2006.
- [11] S. Bekker-Jensen, J. R. Danielsen, K. Fugger, I. Gromova, A. Nerstedt, C. Lukas, J. Bartek, J. Lukas, and N. Mailand. HERC2 coordinates ubiquitin-dependent assembly of DNA repair factors on damaged chromosomes. *Nat Cell Biol*, 12(1):80–86, 2010. 10.1038/ncb2008.
- [12] S. Bekker-Jensen and N. Mailand. Assembly and function of DNA double-strand break repair foci in mammalian cells. *DNA Repair*, 9(12):1219–1228, 2010.
- [13] F. E. Benson, A. Stasiak, and S. C. West. Purification and characterization of the human Rad51 protein, an analogue of *E. coli* RecA. *EMBO J*, 13(23):5764–71, 1994.
- [14] E. Bernstein, A. A. Caudy, S. M. Hammond, and G. J. Hannon. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*, 409(6818):363–6, 2001.
- [15] S. Bhalla, S. Balasubramanian, K. David, M. Sirisawad, J. Buggy, L. Mauro, S. Prachand, R. Miller, L. I. Gordon, and A. M. Evens. PCI-24781 induces caspase and reactive oxygen species-dependent apoptosis through NF-kappaB mechanisms and is synergistic with bortezomib in lymphoma cells. *Clin Cancer Res*, 15(10):3354–65, 2009.

- [16] P. Bork, N. Blomberg, and M. Nilges. Internal repeats in the BRCA2 protein sequence. *Nat Genet*, 13(1):22–3, 1996.
- [17] F. Bouquet, M. Ousset, D. Biard, F. Fallone, S. Dauvillier, P. Frit, B. Salles, and C. Muller. A DNA-dependent stress response involving DNA-PK occurs in hypoxic cells and contributes to cellular adaptation to hypoxia. *J Cell Sci*, 124(Pt 11):1943–51, 2011.
- [18] J.-M. Brondello, M. J. Pillaire, C. Rodriguez, P.-A. Gourraud, J. Selves, C. Cazaux, and J. Piette. Novel evidences for a tumor suppressor role of Rev3, the catalytic subunit of Pol zeta. *Oncogene*, 27(47):6093–101, 2008.
- [19] H. E. Bryant, N. Schultz, H. D. Thomas, K. M. Parker, D. Flower, E. Lopez, S. Kyle, M. Meuth, N. J. Curtin, and T. Helleday. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature*, 434(7035):913–917, 2005.
- [20] C. T. Carson, R. A. Schwartz, T. H. Stracker, C. E. Lilley, D. V. Lee, and M. D. Weitzman. The Mre11 complex is required for ATM activation and the G2/M checkpoint. *EMBO J*, 22(24):6610–20, 2003.
- [21] J. Cervenka, D. Arthur, and C. Yasis. Mitomycin C test for diagnostic differentiation of idiopathic aplastic anemia and fanconi anemia. *Pediatrics*, 67(1):119–27, 1981.

- [22] R. S. Chaganti, S. Schonberg, and J. German. A manyfold increase in sister chromatid exchanges in Bloom's syndrome lymphocytes. *Proc Natl Acad Sci U S A*, 71(11):4508–12, 1974.
- [23] Q. M. Chen, J. C. Bartholomew, J. Campisi, M. Acosta, J. D. Reagan, and B. N. Ames. Molecular analysis of H₂O₂-induced senescent-like growth arrest in normal human fibroblasts: p53 and Rb control G1 arrest but not cell replication. *Biochem J*, 332 (Pt 1):43–50, 1998.
- [24] P. Chi, S. Van Komen, M. G. Sehorn, S. Sigurdsson, and P. Sung. Roles of ATP binding and ATP hydrolysis in human Rad51 recombinase function. *DNA Repair*, 5(3):381–91, 2006.
- [25] A. Choudhury, H. Zhao, F. Jalali, S. Al Rashid, J. Ran, S. Supiot, A. E. Kiltie, and R. G. Bristow. Targeting homologous recombination using imatinib results in enhanced tumor cell chemosensitivity and radiosensitivity. *Mol Cancer Ther*, 8(1):203–13, 2009.
- [26] A. Ciccia, A. Constantinou, and S. C. West. Identification and characterization of the human mus81-eme1 endonuclease. *J Biol Chem*, 278(27):25172–8, 2003.
- [27] A. Ciccia and S. J. Elledge. The DNA damage response: making it safe to play with knives. *Mol Cell*, 40(2):179–204, 2010.
- [28] B. Connolly, C. A. Parsons, F. E. Benson, H. J. Dunderdale, G. J. Sharples, R. G. Lloyd, and S. C. West. Resolution of holliday junctions

- in vitro requires the *Escherichia coli* *ruvC* gene product. *Proc Natl Acad Sci U S A*, 88(14):6063–7, 1991.
- [29] F. Connor, D. Bertwistle, P. J. Mee, G. M. Ross, S. Swift, E. Grigorieva, V. L. Tybulewicz, and A. Ashworth. Tumorigenesis and a DNA repair defect in mice with a truncating *Brca2* mutation. *Nat Genet*, 17(4):423–30, 1997.
- [30] A. Constantinou, M. Tarsounas, J. K. Karow, R. M. Brosh, V. A. Bohr, I. D. Hickson, and S. C. West. Werner’s syndrome protein (WRN) migrates Holliday junctions and co-localizes with RPA upon replication arrest. *EMBO Rep*, 1(1):80–4, 2000.
- [31] C. Cotta-Ramusino, E. R. McDonald, K. Hurov, M. E. Sowa, J. W. Harper, and S. J. Elledge. A DNA damage response screen identifies RHINO, a 9-1-1 and TopBP1 interacting protein required for ATR signaling. *Science*, 332(6035):1313–7, 2011.
- [32] Y. Dai and S. Grant. New insights into checkpoint kinase 1 in the DNA damage response signaling network. *Clin Cancer Res*, 16(2):376–83, 2010.
- [33] A. D. D’Andrea. The Fanconi Anemia/BRCA signaling pathway: disruption in cisplatin-sensitive ovarian cancers. *Cell Cycle*, 2(4):290–2, 2003.

- [34] S. L. Davies, P. S. North, and I. D. Hickson. Role for BLM in replication-fork restart and suppression of origin firing after replicative stress. *Nat Struct Mol Biol*, 14(7):677–9, 2007.
- [35] M. de Jager, J. van Noort, D. C. van Gent, C. Dekker, R. Kanaar, and C. Wyman. Human Rad50/Mre11 is a flexible complex that can tether DNA ends. *Mol Cell*, 8(5):1129–35, 2001.
- [36] A. J. Deans and S. C. West. FANCM connects the genome instability disorders Bloom’s syndrome and Fanconi anemia. *Mol Cell*, 36(6):943–53, 2009.
- [37] A. J. Deans and S. C. West. DNA interstrand crosslink repair and cancer. *Nat Rev Cancer*, 11(7):467–80, 2011.
- [38] C. Dinant and M. S. Luijsterburg. The emerging role of HP1 in the DNA damage response. *Mol Cell Biol*, 29(24):6335–6340, 2009.
- [39] R. A. DiTullio, Jr, T. A. Mochan, M. Venere, J. Bartkova, M. Sehested, J. Bartek, and T. D. Halazonetis. 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer. *Nat Cell Biol*, 4(12):998–1002, 2002.
- [40] A. Dmochowska, K. Kalita, M. Krawczyk, P. Golik, K. Mroczek, J. Lazowska, P. P. Stepień, and E. Bartnik. A human putative Suv3-like RNA helicase is conserved between *Rhodobacter* and all eukaryotes. *Acta Biochim Pol*, 46(1):155–62, 1999.

- [41] C. Doil, N. Mailand, S. Bekker-Jensen, P. Menard, D. H. Larsen, R. Pepperkok, J. Ellenberg, S. Panier, D. Durocher, J. Bartek, J. Lukas, and C. Lukas. RNF168 binds and amplifies ubiquitin conjugates on damaged chromosomes to allow accumulation of repair proteins. *Cell*, 136(3):435–446, 2009.
- [42] V. Dulić, E. Lees, and S. I. Reed. Association of human cyclin E with a periodic G1-S phase protein kinase. *Science*, 257(5078):1958–61, 1992.
- [43] A. Dupré, L. Boyer-Chatenet, R. M. Sattler, A. P. Modi, J.-H. Lee, M. L. Nicolette, L. Kopelovich, M. Jasin, R. Baer, T. T. Paull, and J. Gautier. A forward chemical genetic screen reveals an inhibitor of the Mre11-Rad50-Nbs1 complex. *Nat Chem Biol*, 4(2):119–25, 2008.
- [44] S. M. Elbashir, W. Lendeckel, and T. Tuschl. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev*, 15(2):188–200, 2001.
- [45] H. Erkkö, B. Xia, J. Nikkila, J. Schleutker, K. Syrjäkoski, A. Mannermaa, A. Kallioniemi, K. Pylkos, S. M. Karppinen, K. Rapakko, A. Miron, Q. Sheng, G. Li, H. Mattila, D. W. Bell, D. A. Haber, M. Grip, M. Reiman, A. Jukkola-Vuorinen, A. Mustonen, J. Kere, L. A. Aaltonen, V. M. Kosma, V. Kataja, Y. Soini, R. I. Drapkin, D. M. Livingston, and R. Winqvist. A recurrent mutation in PALB2 in Finnish cancer families. *Nature*, 446(7133):316–9, 2007.

- [46] J. Falck, J. Coates, and S. P. Jackson. Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. *Nature*, 434(7033):605–11, 2005.
- [47] J. Falck, N. Mailand, R. G. Syljuåsen, J. Bartek, and J. Lukas. The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. *Nature*, 410(6830):842–7, 2001.
- [48] H. Farmer, N. McCabe, C. J. Lord, A. N. Tutt, D. A. Johnson, T. B. Richardson, M. Santarosa, K. J. Dillon, I. Hickson, C. Knights, N. M. Martin, S. P. Jackson, G. C. Smith, and A. Ashworth. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature*, 434(7035):917–21, 2005.
- [49] C. Feijoo, C. Hall-Jackson, R. Wu, D. Jenkins, J. Leitch, D. M. Gilbert, and C. Smythe. Activation of mammalian Chk1 during DNA replication arrest: a role for Chk1 in the intra-S phase checkpoint monitoring replication origin firing. *J Cell Biol*, 154(5):913–23, 2001.
- [50] S. Fekairi, S. Scaglione, C. Chahwan, E. R. Taylor, A. Tissier, S. Coulon, M. Q. Dong, C. Ruse, J. R. Yates, P. Russell, R. P. Fuchs, C. H. McGowan, and P. H. Gaillard. Human SLX4 is a holliday junction resolvase subunit that binds multiple DNA repair/recombination endonucleases. *Cell*, 138(1):78–89, 2009.

- [51] Z. Feng, S. P. Scott, W. Bussen, G. G. Sharma, G. Guo, T. K. Pandita, and S. N. Powell. Rad52 inactivation is synthetically lethal with BRCA2 deficiency. *Proc Natl Acad Sci U S A*, 108(2):686–91, 2011.
- [52] A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, 391(6669):806–11, 1998.
- [53] P. C. Fong, D. S. Boss, T. A. Yap, A. Tutt, P. Wu, M. Mergui-Roelvink, P. Mortimer, H. Swaisland, A. Lau, M. J. O’Connor, A. Ashworth, J. Carmichael, S. B. Kaye, J. H. Schellens, and J. S. de Bono. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N Engl J Med*, 361(2):123–34, 2009.
- [54] Y. Galanty, R. Belotserkovskaya, J. Coates, S. Polo, K. M. Miller, and S. P. Jackson. Mammalian SUMO E3-ligases PIAS1 and PIAS4 promote responses to DNA double-strand breaks. *Nature*, 462(7275):935–939, 2009.
- [55] V. Garcia, S. E. L. Phelps, S. Gray, and M. J. Neale. Bidirectional resection of DNA double-strand breaks by Mre11 and Exo1. *Nature*, 479(7372):241–4, 2011.
- [56] I. Garcia-Higuera, T. Taniguchi, S. Ganesan, M. S. Meyn, C. Timmers, J. Hejna, M. Grompe, and A. D. D’Andrea. Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol Cell*, 7(2):249–62, 2001.

- [57] K. Gari, C. Dacaillet, A. Z. Stasiak, A. Stasiak, and A. Constantinou. The fanconi anemia protein FANCM can promote branch migration of Holliday junctions and replication forks. *Mol Cell*, 29(1):141–8, 2008.
- [58] A. A. Goodarzi, P. Jeggo, and M. Lobrich. The influence of heterochromatin on DNA double strand break repair: Getting the strong, silent type to relax. *DNA Repair*, 9(12):1273–1282, 2010.
- [59] V. G. Gorgoulis, L. V. Vassiliou, P. Karakaidos, P. Zacharatos, A. Kotsinas, T. Liloglou, M. Venere, R. A. Ditullio, N. G. Kastri-nakis, B. Levy, D. Kletsas, A. Yoneta, M. Herlyn, C. Kittas, and T. D. Halazonetis. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature*, 434(7035):907–13, 2005.
- [60] T. M. Gottlieb and S. P. Jackson. The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen. *Cell*, 72(1):131–42, 1993.
- [61] S. Gravel, J. R. Chapman, C. Magill, and S. P. Jackson. DNA helicases Sgs1 and BLM promote DNA double-strand break resection. *Genes Dev*, 22(20):2767–72, 2008.
- [62] T.-P. Gu, F. Guo, H. Yang, H.-P. Wu, G.-F. Xu, W. Liu, Z.-G. Xie, L. Shi, X. He, S.-g. Jin, K. Iqbal, Y. G. Shi, Z. Deng, P. E. Szabó, G. P. Pfeifer, J. Li, and G.-L. Xu. The role of Tet3 DNA dioxygenase

- in epigenetic reprogramming by oocytes. *Nature*, 477(7366):606–10, 2011.
- [63] J. F. Haince, S. Kozlov, V. L. Dawson, T. M. Dawson, M. J. Hendzel, M. F. Lavin, and G. G. Poirier. Ataxia telangiectasia mutated (ATM) signaling network is modulated by a novel poly(ADP-ribose)-dependent pathway in the early response to DNA-damaging agents. *J Biol Chem*, 282(22):16441–53, 2007.
- [64] J. F. Haince, D. McDonald, A. Rodrigue, U. Dury, J. Y. Masson, M. J. Hendzel, and G. G. Poirier. PARP1-dependent kinetics of recruitment of MRE11 and NBS1 proteins to multiple DNA damage sites. *J Biol Chem*, 283(2):1197–208, 2008.
- [65] M. Hall, S. Misra, M. Chaudhuri, and G. Chaudhuri. Peptide aptamer mimicking RAD51-binding domain of BRCA2 inhibits DNA damage repair and survival in *Trypanosoma brucei*. *Microb Pathog*, 50(5):252–62, 2011.
- [66] O. Hammarsten and G. Chu. DNA-dependent protein kinase: DNA binding and activation in the absence of ku. *Proc Natl Acad Sci U S A*, 95(2):525–530, 1998.
- [67] K. Hanada, M. Budzowska, M. Modesti, A. Maas, C. Wyman, J. Essers, and R. Kanaar. The structure-specific endonuclease Mus81-Eme1 promotes conversion of interstrand DNA crosslinks into double-strands breaks. *EMBO J*, 25(20):4921–32, 2006.

- [68] L. T. Hansen, C. Lundin, M. Spang-Thomsen, L. N. Petersen, and T. Helleday. The role of RAD51 in etoposide (VP16) resistance in small cell lung cancer. *Int J Cancer*, 105(4):472–9, 2003.
- [69] J. W. Harper and S. J. Elledge. The DNA damage response: ten years after. *Mol Cell*, 28(5):739–45, 2007.
- [70] E. Hartsuiker, M. J. Neale, and A. M. Carr. Distinct requirements for the Rad32(Mre11) nuclease and Ctp1(CtIP) in the removal of covalently bound topoisomerase I and II from DNA. *Mol Cell*, 33(1):117–23, 2009.
- [71] T. Helleday. Homologous recombination in cancer development, treatment and development of drug resistance. *Carcinogenesis*, 31(6):955–960, 2010.
- [72] T. Helleday, E. Petermann, C. Lundin, B. Hodgson, and R. A. Sharma. DNA repair pathways as targets for cancer therapy. *Nat Rev Cancer*, 8(3):193–204, 2008.
- [73] A. Hershko and A. Ciechanover. The ubiquitin system for protein degradation. *Annu Rev Biochem*, 61:761–807, 1992.
- [74] H. Hohegger, D. Dejsuphong, T. Fukushima, C. Morrison, E. Sonoda, V. Schreiber, G. Y. Zhao, A. Saberi, M. Masutani, N. Adachi, H. Koyama, G. de Murcia, and S. Takeda. Parp-1 protects homologous

- recombination from interference by Ku and Ligase IV in vertebrate cells. *EMBO J*, 25(6):1305–14, 2006.
- [75] W. K. Holloman. Unraveling the mechanism of BRCA2 in homologous recombination. *Nat Struct Mol Biol*, 18(7):748–54, 2011.
- [76] K. P. Hopfner, A. Karcher, L. Craig, T. T. Woo, J. P. Carney, and J. A. Tainer. Structural biochemistry and interaction architecture of the DNA double-strand break repair Mre11 nuclease and Rad50-ATPase. *Cell*, 105(4):473–85, 2001.
- [77] N. G. Howlett, T. Taniguchi, S. Olson, B. Cox, Q. Waisfisz, C. De Die-Smulders, N. Persky, M. Grompe, H. Joenje, G. Pals, H. Ikeda, E. A. Fox, and A. D. D’Andrea. Biallelic inactivation of BRCA2 in Fanconi anemia. *Science*, 297(5581):606–9, 2002.
- [78] Y. H. Hsiang, M. G. Lihou, and L. F. Liu. Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin. *Cancer Res*, 49(18):5077–82, 1989.
- [79] Z. Hu, I. Gomes, S. K. Horrigan, J. Kravarusic, B. Mar, Z. Arbieva, B. Chyna, N. Fulton, S. Edassery, A. Raza, and C. A. Westbrook. A novel nuclear protein, 5qNCA (LOC51780) is a candidate for the myeloid leukemia tumor suppressor gene on chromosome 5 band q31. *Oncogene*, 20(47):6946–54, 2001.

- [80] F. Huang, N. A. Motlekar, C. M. Burgwin, A. D. Napper, S. L. Diamond, and A. V. Mazin. Identification of specific inhibitors of human RAD51 recombinase using high-throughput screening. *ACS Chem Bio*, 6(6):628–635, 2011.
- [81] M. Huang, J. M. Kim, B. Shiotani, K. Yang, L. Zou, and A. D. D’Andrea. The FANCM/FAAP24 complex is required for the DNA interstrand crosslink-induced checkpoint response. *Mol Cell*, 39(2):259–68, 2010.
- [82] Y. Huang, S. Nakada, T. Ishiko, T. Utsugisawa, R. Datta, S. Kharbanda, K. Yoshida, R. V. Talanian, R. Weichselbaum, D. Kufe, and Z. M. Yuan. Role for caspase-mediated cleavage of Rad51 in induction of apoptosis by DNA damage. *Mol Cell Biol*, 19(4):2986–97, 1999.
- [83] M. S. Y. Huen, R. Grant, I. Manke, K. Minn, X. Yu, M. B. Yaffe, and J. Chen. RNF8 transduces the DNA-damage signal via histone ubiquitylation and checkpoint protein assembly. *Cell*, 131(5):901–914, 2007.
- [84] P. Huertas and S. P. Jackson. Human CtIP mediates cell cycle control of DNA end resection and double strand break repair. *J Biol Chem*, 284(14):9558–65, 2009.
- [85] K. E. Hurov, C. Cotta-Ramusino, and S. J. Elledge. A genetic screen identifies the Triple T complex required for DNA damage signaling and ATM and ATR stability. *Genes Dev*, 24(17):1939–50, 2010.

- [86] S. C. Ip, U. Rass, M. G. Blanco, H. R. Flynn, J. M. Skehel, and S. C. West. Identification of Holliday junction resolvases from humans and yeast. *Nature*, 456(7220):357–61, 2008.
- [87] R. Ishida and M. Buchwald. Susceptibility of fanconi’s anemia lymphoblasts to dna-cross-linking and alkylating agents. *Cancer Res*, 42(10):4000–6, 1982.
- [88] H. Iwasaki, M. Takahagi, T. Shiba, A. Nakata, and H. Shinagawa. Escherichia coli RuvC protein is an endonuclease that resolves the holliday structure. *EMBO J*, 10(13):4381–9, 1991.
- [89] V. N. Iyer and W. Szybalski. Mitomycins and porfiromycin: chemical mechanism of activation and cross-linking of DNA. *Science*, 145(3627):55–8, 1964.
- [90] A. Jazayeri, J. Falck, C. Lukas, J. Bartek, G. C. Smith, J. Lukas, and S. P. Jackson. ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nat Cell Biol*, 8(1):37–45, 2006.
- [91] P. A. Jeggo, V. Geuting, and M. Labrich. The role of homologous recombination in radiation-induced double-strand break repair. *Radiother Oncol*, 101(1):7–12, 2011.

- [92] R. B. Jensen, A. Carreira, and S. C. Kowalczykowski. Purified human BRCA2 stimulates RAD51-mediated recombination. *Nature*, 467(7316):678–83, 2010.
- [93] S. Jirawatnotai, Y. Hu, W. Michowski, J. E. Elias, L. Becks, F. Bienvenu, A. Zagozdzon, T. Goswami, Y. E. Wang, A. B. Clark, T. A. Kunkel, T. van Harn, B. Xia, M. Correll, J. Quackenbush, D. M. Livingston, S. P. Gygi, and P. Sicinski. A function for cyclin D1 in DNA repair uncovered by protein interactome analyses in human cancers. *Nature*, 474(7350):230–4, 2011.
- [94] S. K. Kachhap, N. Rosmus, S. J. Collis, M. S. Q. Kortenhorst, M. D. Wissing, M. Hedayati, S. Shabbeer, J. Mendonca, J. Deangelis, L. Marchionni, J. Lin, N. Hati, J. W. R. Nortier, T. L. DeWeese, H. Hammers, and M. A. Carducci. Downregulation of homologous recombination DNA repair genes by HDAC inhibition in prostate cancer is mediated through the E2F1 transcription factor. *PLoS ONE*, 5(6):e11208, 2010.
- [95] A. Kaidi, B. T. Weinert, C. Choudhary, and S. P. Jackson. Human SIRT6 promotes DNA end resection through CtIP deacetylation. *Science*, 329(5997):1348–1353, 2010.
- [96] Y. Kee and A. D. D’Andrea. Expanded roles of the Fanconi anemia pathway in preserving genomic stability. *Genes Dev*, 24(16):1680–94, 2010.

- [97] L. Khidr, G. Wu, A. Davila, V. Procaccio, D. Wallace, and W.-H. Lee. Role of SUV3 helicase in maintaining mitochondrial homeostasis in human cells. *J Biol Chem*, 283(40):27064–27073, 2008.
- [98] S. Kim, D. H. Jun, H. J. Kim, K.-C. Jeong, and C.-H. Lee. Development of a high-content screening method for chemicals modulating DNA damage response. *J Biomol Screen*, 16(2):259–65, 2011.
- [99] Y. Kim, F. P. Lach, R. Desetty, H. Hanenberg, A. D. Auerbach, and A. Smogorzewska. Mutations of the SLX4 gene in Fanconi anemia. *Nat Genet*, 43(2):142–6, 2011.
- [100] J. C. Ko, S. C. Ciou, C. M. Cheng, L. H. Wang, J. H. Hong, M. Y. Jheng, S. T. Ling, and Y. W. Lin. Involvement of Rad51 in cytotoxicity induced by epidermal growth factor receptor inhibitor (gefitinib, IressaR) and chemotherapeutic agents in human lung cancer cells. *Carcinogenesis*, 29(7):1448–58, 2008.
- [101] C. A. Koch, R. Agyei, S. Galicia, P. Metalnikov, P. O’Donnell, A. Starostine, M. Weinfeld, and D. Durocher. Xrcc4 physically links DNA end processing by polynucleotide kinase to DNA ligation by DNA ligase IV. *EMBO J*, 23(19):3874–3885, 2004.
- [102] E. Kodym, R. Kodym, A. E. Reis, A. A. Habib, M. D. Story, and D. Saha. The small-molecule CDK inhibitor, SNS-032, enhances cellular radiosensitivity in quiescent and hypoxic non-small cell lung cancer cells. *Lung Cancer*, 66(1):37–47, 2009.

- [103] N. K. Kolas, J. R. Chapman, S. Nakada, J. Ylanko, R. Chahwan, F. D. Sweeney, S. Panier, M. Mendez, J. Wildenhain, T. M. Thomson, L. Pelletier, S. P. Jackson, and D. Durocher. Orchestration of the DNA-damage response by the RNF8 ubiquitin ligase. *Science*, 318(5856):1637–1640, 2007.
- [104] S. Kotian, S. Liyanarachchi, A. Zelent, and J. D. Parvin. Histone deacetylases 9 and 10 are required for homologous recombination. *J Biol Chem*, 286(10):7722–6, 2011.
- [105] K. Kratz, B. Schopf, S. Kaden, A. Sandoel, R. Eberhard, C. Lademann, E. Cannav, A. A. Sartori, M. O. Hengartner, and J. Jiricny. Deficiency of FANCD2-associated nuclease KIAA1018/FAN1 sensitizes cells to interstrand crosslinking agents. *Cell*, 142(1):77–88, 2010.
- [106] E. Krausz. High-content siRNA screening. *Mol Biosyst*, 3(4):232–40, 2007.
- [107] A. Kurimasa, S. Kumano, N. V. Boubnov, M. D. Story, C.-S. Tung, S. R. Peterson, and D. J. Chen. Requirement for the kinase activity of human DNA-dependent protein kinase catalytic subunit in DNA strand break rejoining. *Mol Cell Biol*, 19(5):3877–3884, 1999.
- [108] B. E. Lally, G. A. Geiger, S. Kridel, A. E. Arcury-Quandt, M. E. Robbins, N. D. Kock, K. Wheeler, P. Peddi, A. Georgakilas, G. D. Kao, and C. Koumenis. Identification and biological evaluation of a

- novel and potent small molecule radiation sensitizer via an unbiased screen of a chemical library. *Cancer Res*, 67(18):8791–9, 2007.
- [109] B. J. Lamarche, N. I. Orazio, and M. D. Weitzman. The MRN complex in double-strand break repair and telomere maintenance. *FEBS Lett*, 584(17):3682–95, 2010.
 - [110] P. Langerak, E. Mejia-Ramirez, O. Limbo, and P. Russell. Release of Ku and MRN from DNA ends by Mre11 nuclease activity and Ctp1 is required for homologous recombination repair of double-strand breaks. *PLoS Genet*, 7(9):e1002271, 2011.
 - [111] J. H. Lee and T. T. Paull. ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. *Science*, 308(5721):551–4, 2005.
 - [112] L. Li, H. Wang, E. S. Yang, C. L. Arteaga, and F. Xia. Erlotinib attenuates homologous recombinational repair of chromosomal breaks in human breast cancer cells. *Cancer Res*, 68(22):9141–6, 2008.
 - [113] M. R. Lieber. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annu Rev Biochem*, 79(1):181–211, 2010.
 - [114] R. Litman, M. Peng, Z. Jin, F. Zhang, J. Zhang, S. Powell, P. R. Andreassen, and S. B. Cantor. BACH1 is critical for homologous recom-

- bination and appears to be the Fanconi anemia gene product FANCD1.
Cancer Cell, 8(3):255–65, 2005.
- [115] F. Liu and W. H. Lee. CtIP activates its own and cyclin D1 promoters via the E2F/RB pathway during G1/S progression. *Mol Cell Biol*, 26(8):3124–34, 2006.
- [116] H. Liu, C. Zang, J.-H. Schefe, S. Schwarzlose-Schwarck, A.-C. Regierer, E. Elstner, C.-O. Schulz, C. Scholz, K. Possinger, and J. Eucker. The mTOR inhibitor RAD001 sensitizes tumor cells to the cytotoxic effect of carboplatin in breast cancer in vitro. *Anticancer Res*, 31(9):2713–22, 2011.
- [117] J. Liu, T. Doty, B. Gibson, and W. D. Heyer. Human BRCA2 protein promotes RAD51 filament formation on RPA-covered single-stranded DNA. *Nat Struct Mol Biol*, 17(10):1260–2, 2010.
- [118] Y. Liu, M. Tarsounas, P. O’regan, and S. C. West. Role of RAD51C and XRCC3 in genetic recombination and DNA repair. *J Biol Chem*, 282(3):1973–9, 2007.
- [119] B. Lomenick, R. W. Olsen, and J. Huang. Identification of direct protein targets of small molecules. *ACS Chem Biol*, 6(1):34–46, 2011.
- [120] G. Lopez, J. Liu, W. Ren, W. Wei, S. Wang, G. Lahat, Q. S. Zhu, W. G. Bornmann, D. J. McConkey, R. E. Pollock, and D. C. Lev. Combining PCI-24781, a novel histone deacetylase inhibitor, with chemotherapy

- for the treatment of soft tissue sarcoma. *Clin Cancer Res*, 15(10):3472–83, 2009.
- [121] C. J. Lord, S. McDonald, S. Swift, N. C. Turner, and A. Ashworth. A high-throughput RNA interference screen for DNA repair determinants of PARP inhibitor sensitivity. *DNA Repair*, 7(12):2010–9, 2008.
- [122] Y. Ma, H. Lu, B. Tippin, M. F. Goodman, N. Shimazaki, O. Koiwai, C.-L. Hsieh, K. Schwarz, and M. R. Lieber. A biochemically defined system for mammalian nonhomologous DNA end joining. *Mol Cell*, 16(5):701–713, 2004.
- [123] C. MacKay, A.-C. Daclais, C. Lundin, A. Agostinho, A. J. Deans, T. J. MacArtney, K. Hofmann, A. Gartner, S. C. West, T. Helleday, D. M. J. Lilley, and J. Rouse. Identification of KIAA1018/FAN1, a dna repair nuclease recruited to dna damage by monoubiquitinated FANCD2. *Cell*, 142(1):65–76, 2010.
- [124] R. N. MacKinnon, G. Kannourakis, M. Wall, and L. J. Campbell. A cryptic deletion in 5q31.2 provides further evidence for a minimally deleted region in myelodysplastic syndromes. *Cancer Genet*, 204(4):187–94, 2011.
- [125] L. Maggiorella, E. Deutsch, V. Frascogna, N. Chavaudra, L. Jeanson, F. Milliat, F. Eschwege, and J. Bourhis. Enhancement of radiation response by roscovitine in human breast carcinoma in vitro and in vivo. *Cancer Res*, 63(10):2513–7, 2003.

- [126] B. L. Mahaney, K. Meek, and S. P. Lees-miller. Repair of ionizing radiation-induced DNA double-strand breaks by non-homologous end-joining. *Biochem J*, 417(3):639–650, 2009.
- [127] N. Mailand, S. Bekker-Jensen, H. Faustrup, F. Melander, J. Bartek, C. Lukas, and J. Lukas. RNF8 ubiquitylates histones at DNA double-strand breaks and promotes assembly of repair proteins. *Cell*, 131(5):887–900, 2007.
- [128] N. J. Marston, W. J. Richards, D. Hughes, D. Bertwistle, C. J. Marshall, and A. Ashworth. Interaction between the product of the breast cancer susceptibility gene BRCA2 and DSS1, a protein functionally conserved from yeast to mammals. *Mol Cell Biol*, 19(7):4633–42, 1999.
- [129] P. Matulova, V. Marini, R. C. Burgess, A. Sisakova, Y. Kwon, R. Rothstein, P. Sung, and L. Krejci. Co-operativity of Mus81-Mms4 with Rad54 in the resolution of recombination and replication intermediates. *J Biol Chem*, 284(12):7733–45, 2009.
- [130] A. V. Mazin, O. M. Mazina, D. V. Bugreev, and M. J. Rossi. Rad54, the motor of homologous recombination. *DNA Repair*, 9(3):286–302, 2010.
- [131] A. R. Meetei, A. L. Medhurst, C. Ling, Y. Xue, T. R. Singh, P. Bier, J. Steltenpool, S. Stone, I. Dokal, C. G. Mathew, M. Hoatlin, H. Joenje, J. P. de Winter, and W. Wang. A human ortholog of archaeal DNA re-

- pair protein Hef is defective in Fanconi anemia complementation group M. *Nat Genet*, 37(9):958–63, 2005.
- [132] F. Melander, S. Bekker-Jensen, J. Falck, J. Bartek, N. Mailand, and J. Lukas. Phosphorylation of SDT repeats in the MDC1 N terminus triggers retention of NBS1 at the DNA damage modified chromatin. *J Cell Biol*, 181(2):213–226, 2008.
- [133] T. Menzel, V. Nohse-Kumpf, A. N. Kousholt, D. K. Klein, C. Lund-Andersen, M. Lees, J. V. Johansen, R. G. Syljuvsen, and C. S. Sorensen. A genetic screen identifies BRCA2 and PALB2 as key regulators of G2 checkpoint maintenance. *EMBO Rep*, 12(7):705–12, 2011.
- [134] A. Mikhailov, M. Shinohara, and C. L. Rieder. Topoisomerase II and histone deacetylase inhibitors delay the G2/M transition by triggering the p38 MAPK checkpoint pathway. *J Cell Biol*, 166(4):517–26, 2004.
- [135] M. Minczuk, S. Mroczek, S. D. Pawlak, and P. P. Stepień. Human ATP-dependent RNA/DNA helicase hSuv3p interacts with the cofactor of survivin HBXIP. *FEBS J*, 272(19):5008–5019, 2005.
- [136] M. Minczuk, J. Piwowarski, M. A. Papworth, K. Awiszus, S. Schalin-ski, A. Dziembowski, A. Dmochowska, E. Bartnik, K. Tokatlidis, P. P. Stepień, and P. Borowski. Localisation of the human hSuv3p helicase in the mitochondrial matrix and its preferential unwinding of dsDNA. *Nucleic Acids Res*, 30(23):5074–86, 2002.

- [137] O. K. Mirzoeva and J. H. Petrini. DNA damage-dependent nuclear dynamics of the Mre11 complex. *Mol Cell Biol*, 21(1):281–8, 2001.
- [138] Y. Mishima, S. Miyagi, A. Saraya, M. Negishi, M. Endoh, T. A. Endo, T. Toyoda, J. Shinga, T. Katsumoto, T. Chiba, N. Yamaguchi, I. Kitabayashi, H. Koseki, and A. Iwama. The Hbo1-Brd1/Brpf2 complex is responsible for global acetylation of H3K14 and required for fetal liver erythropoiesis. *Blood*, 118(9):2443–53, 2011.
- [139] K. Mizuuchi, B. Kemper, J. Hays, and R. A. Weisberg. T4 endonuclease VII cleaves holliday structures. *Cell*, 29(2):357–65, 1982.
- [140] S. Modi, A. Stopeck, H. Linden, D. Solit, S. Chandarlapaty, N. Rosen, G. D’Andrea, M. Dickler, M. E. Moynahan, S. Sugarman, W. Ma, S. Patil, L. Norton, A. L. Hannah, and C. Hudis. HSP90 inhibition is effective in breast cancer: A Phase II trial of Tanespimycin (17-aag) plus Trastuzumab in patients with HER2-positive metastatic breast cancer progressing on Trastuzumab. *Clin Cancer Res*, 17(15):5132–5139, 2011.
- [141] M. A. Morgan, L. A. Parsels, L. Zhao, J. D. Parsels, M. A. Davis, M. C. Hassan, S. Arumugarajah, L. Hylander-Gans, D. Morosini, D. M. Simeone, C. E. Canman, D. P. Normolle, S. D. Zabludoff, J. Maybaum, and T. S. Lawrence. Mechanism of radiosensitization by the Chk1/2 inhibitor AZD7762 involves abrogation of the G2 checkpoint

- and inhibition of homologous recombinational DNA repair. *Cancer Res*, 70(12):4972–4981, 2010.
- [142] M. Morimatsu, G. Donoho, and P. Hasty. Cells deleted for Brca2 COOH terminus exhibit hypersensitivity to gamma-radiation and premature senescence. *Cancer Res*, 58(15):3441–7, 1998.
- [143] J. R. Morris, C. Boutell, M. Keppler, R. Densham, D. Weekes, A. Alamshah, L. Butler, Y. Galanty, L. Pangon, T. Kiuchi, T. Ng, and E. Solomon. The SUMO modification pathway is involved in the BRCA1 response to genotoxic stress. *Nature*, 462(7275):886–890, 2009.
- [144] C. Morrison, A. Shinohara, E. Sonoda, Y. Yamaguchi-Iwai, M. Takata, R. R. Weichselbaum, and S. Takeda. The essential functions of human Rad51 are independent of ATP hydrolysis. *Mol Cell Biol*, 19(10):6891–7, 1999.
- [145] G. Mosedale, W. Niedzwiedz, A. Alpi, F. Perrina, J. B. Pereira-Leal, M. Johnson, F. Langevin, P. Pace, and K. J. Patel. The vertebrate Hef ortholog is a component of the Fanconi anemia tumor-suppressor pathway. *Nat Struct Mol Biol*, 12(9):763–71, 2005.
- [146] A. Mukherji, V. C. Janbandhu, and V. Kumar. GSK-3beta-dependent destabilization of cyclin D1 mediates replicational stress-induced arrest of cell cycle. *FEBS Lett*, 582(7):1111–6, 2008.

- [147] J. R. Mullen, V. Kaliraman, S. S. Ibrahim, and S. J. Brill. Requirement for three novel protein complexes in the absence of the Sgs1 DNA helicase in *Saccharomyces cerevisiae*. *Genetics*, 157(1):103–18, 2001.
- [148] P. A. Muniandy, J. Liu, A. Majumdar, S.-t. Liu, and M. M. Seidman. DNA interstrand crosslink repair in mammalian cells: step by step. *Crit Rev Biochem Mol Biol*, 45(1):23–49, 2010.
- [149] I. M. Muñoz, K. Hain, A. C. Déclais, M. Gardiner, G. W. Toh, L. Sanchez-Pulido, J. M. Heuckmann, R. Toth, T. Macartney, B. Eppink, R. Kanaar, C. P. Ponting, D. M. Lilley, and J. Rouse. Coordination of structure-specific nucleases by human SLX4/BTBD12 is required for DNA repair. *Mol Cell*, 35(1):116–27, 2009.
- [150] Y. Murakawa, E. Sonoda, L. J. Barber, W. Zeng, K. Yokomori, H. Kimura, A. Niimi, A. Lehmann, G. Y. Zhao, H. Hohegger, S. J. Boulton, and S. Takeda. Inhibitors of the proteasome suppress homologous DNA recombination in mammalian cells. *Cancer Res*, 67(18):8536–43, 2007.
- [151] K. Nakamura, T. Kogame, H. Oshiumi, A. Shinohara, Y. Sumitomo, K. Agama, Y. Pommier, K. M. Tsutsui, K. Tsutsui, E. Hartsuiker, T. Ogi, S. Takeda, and Y. Taniguchi. Collaborative action of Brca1 and CtIP in elimination of covalent modifications from double-strand breaks to facilitate subsequent break repair. *PLoS Genet*, 6(1):e1000828, 2010.

- [152] H. Niida, Y. Katsuno, B. Banerjee, M. P. Hande, and M. Nakanishi. Specific role of Chk1 phosphorylations in cell survival and checkpoint activation. *Mol Cell Biol*, 27(7):2572–81, 2007.
- [153] A. V. Nimonkar, J. Genschel, E. Kinoshita, P. Polaczek, J. L. Campbell, C. Wyman, P. Modrich, and S. C. Kowalczykowski. BLM-DNA2-RPA-MRN and EXO1-BLM-RPA-MRN constitute two DNA end resection machineries for human DNA break repair. *Genes Dev*, 25(4):350–62, 2011.
- [154] M. Noguchi, D. Yu, R. Hirayama, Y. Ninomiya, E. Sekine, N. Kubota, K. Ando, and R. Okayasu. Inhibition of homologous recombination repair in irradiated tumor cells pretreated with Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin. *Biochem Biophys Res Commun*, 351(3):658–63, 2006.
- [155] B. Norquist, K. A. Wurz, C. C. Pennil, R. Garcia, J. Gross, W. Sakai, B. Y. Karlan, T. Taniguchi, and E. M. Swisher. Secondary somatic mutations restoring BRCA1/2 predict chemotherapy resistance in hereditary ovarian carcinomas. *J Clin Oncol*, 29(22):3008–15, 2011.
- [156] J. O’Shaughnessy, C. Osborne, J. E. Pippin, M. Yoffe, D. Patt, C. Rocha, I. C. Koo, B. M. Sherman, and C. Bradley. Iniparib plus chemotherapy in metastatic triple-negative breast cancer. *N Engl J Med*, 364(3):205–14, 2011.

- [157] F. Pâques and J. E. Haber. Two pathways for removal of nonhomologous DNA ends during double-strand break repair in *Saccharomyces cerevisiae*. *Mol Cell Biol*, 17(11):6765–71, 1997.
- [158] S.-J. Park, Y.-J. Lee, B. D. Beck, and S.-H. Lee. A positive involvement of RecQL4 in UV-induced S-phase arrest. *DNA Cell Biol*, 25(12):696–703, 2006.
- [159] K. J. Patel, V. P. Yu, H. Lee, A. Corcoran, F. C. Thistlethwaite, M. J. Evans, W. H. Colledge, L. S. Friedman, B. A. Ponder, and A. R. Venkataraman. Involvement of Brca2 in DNA repair. *Mol Cell*, 1(3):347–57, 1998.
- [160] T. T. Paull and M. Gellert. Nbs1 potentiates ATP-driven DNA unwinding and endonuclease cleavage by the Mre11/Rad50 complex. *Genes Dev*, 13(10):1276–88, 1999.
- [161] R. D. Paulsen, D. V. Soni, R. Wollman, A. T. Hahn, M.-C. Yee, A. Guan, J. A. Hesley, S. C. Miller, E. F. Cromwell, D. E. Solow-Cordero, T. Meyer, and K. A. Cimprich. A genome-wide siRNA screen reveals diverse cellular processes and pathways that mediate genome stability. *Mol Cell*, 35(2):228–39, 2009.
- [162] M. Pereira, P. Mason, R. J. Szczesny, L. Maddukuri, S. Dziwura, R. Jędrzejczak, E. Paul, A. Wojcik, L. Dybczynska, B. Tudek, E. Bartnik, J. Klysik, V. A. Bohr, and P. P. Stepien. Interaction of human SUV3

- RNA/DNA helicase with BLM helicase; loss of the SUV3 gene results in mouse embryonic lethality. *Mech Age Dev*, 128(11-12):609–617, 2007.
- [163] E. Petermann and T. Helleday. Pathways of mammalian replication fork restart. *Nat Rev Mol Cell Biol*, 11(10):683–7, 2010.
- [164] P. Pichierri and F. Rosselli. The DNA crosslink-induced s-phase checkpoint depends on ATR-CHK1 and ATR-NBS1-FANCD2 pathways. *EMBO J*, 23(5):1178–87, 2004.
- [165] A. J. Pierce, R. D. Johnson, L. H. Thompson, and M. Jasin. XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. *Genes Dev*, 13(20):2633–2638, 1999.
- [166] N. Rahman, S. Seal, D. Thompson, P. Kelly, A. Renwick, A. Elliott, S. Reid, K. Spanova, R. Barfoot, T. Chagtai, H. Jayatilake, L. McGuffog, S. Hanks, D. G. Evans, D. Eccles, D. F. Easton, M. R. Stratton, and B. C. S. C. (UK). PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. *Nat Genet*, 39(2):165–7, 2007.
- [167] M. Raman, S. Earnest, K. Zhang, Y. Zhao, and M. H. Cobb. TAO kinases mediate activation of p38 in response to DNA damage. *EMBO J*, 26(8):2005–14, 2007.
- [168] S. Reid, D. Schindler, H. Hanenberg, K. Barker, S. Hanks, R. Kalb, K. Neveling, P. Kelly, S. Seal, M. Freund, M. Wurm, S. D. Batish, F. P.

- Lach, S. Yetgin, H. Neitzel, H. Ariffin, M. Tischkowitz, C. G. Mathew, A. D. Auerbach, and N. Rahman. Biallelic mutations in PALB2 cause fanconi anemia subtype FA-N and predispose to childhood cancer. *Nat Genet*, 39(2):162–4, 2007.
- [169] H. C. Reinhardt, A. S. Aslanian, J. A. Lees, and M. B. Yaffe. p53-deficient cells rely on ATM- and ATR-mediated checkpoint signaling through the p38MAPK/MK2 pathway for survival after DNA damage. *Cancer Cell*, 11(2):175–89, 2007.
- [170] E. P. Rogakou, D. R. Pilch, A. H. Orr, V. S. Ivanova, and W. M. Bonner. DNA double-stranded breaks induce histone H2AX Phosphorylation on Serine 139. *J Biol Chem*, 273(10):5858–5868, 1998.
- [171] W. Sakai, E. M. Swisher, B. Y. Karlan, M. K. Agarwal, J. Higgins, C. Friedman, E. Villegas, C. Jacquemont, D. J. Farrugia, F. J. Couch, N. Urban, and T. Taniguchi. Secondary mutations as a mechanism of cisplatin resistance in BRCA2-mutated cancers. *Nature*, 451(7182):1116–20, 2008.
- [172] S. Sakamoto, K. Iijima, D. Mochizuki, K. Nakamura, K. Teshigawara, J. Kobayashi, S. Matsuura, H. Tauchi, and K. Komatsu. Homologous recombination repair is regulated by domains at the N- and C-terminus of NBS1 and is dissociated with ATM functions. *Oncogene*, 26(41):6002–9, 2007.

- [173] D. Salles, A. L. Mencialha, I. C. Ireno, L. Wiesmiller, and E. Abdelhay. BCR-ABL stimulates mutagenic homologous DNA double-strand break repair via the DNA-end-processing factor CtIP. *Carcinogenesis*, 32(1):27–34, 2011.
- [174] J. San Filippo, P. Sung, and H. Klein. Mechanism of eukaryotic homologous recombination. *Annu Rev Biochem*, 77:229–57, 2008.
- [175] J. F. San Miguel, R. Schlag, N. K. Khuageva, M. A. Dimopoulos, O. Shpilberg, M. Kropff, I. Spicka, M. T. Petrucci, A. Palumbo, O. S. Samoilova, A. Dmoszynska, K. M. Abdulkadyrov, R. Schots, B. Jiang, M.-V. Mateos, K. C. Anderson, D. L. Esseltine, K. Liu, A. Cakana, H. van de Velde, P. G. Richardson, and the VISTA Trial Investigators. Bortezomib plus melphalan and prednisone for initial treatment of multiple myeloma. *N Engl J Med*, 359(9):906–917, 2008.
- [176] Y. Sanchez, C. Wong, R. S. Thoma, R. Richman, Z. Wu, H. Piwnicka-Worms, and S. J. Elledge. Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. *Science*, 277(5331):1497–501, 1997.
- [177] A. A. Sartori, C. Lukas, J. Coates, M. Mistrik, S. Fu, J. Bartek, R. Baer, J. Lukas, and S. P. Jackson. Human CtIP promotes DNA end resection. *Nature*, 450(7169):509–14, 2007.

- [178] E. K. Schwartz and W. D. Heyer. Processing of joint molecule intermediates by structure-selective endonucleases during homologous recombination in eukaryotes. *Chromosoma*, 120(2):109–27, 2011.
- [179] S. Sharma, J. K. Hicks, C. L. Chute, J. R. Brennan, J.-Y. Ahn, T. W. Glover, and C. E. Canman. Rev1 and polymerase facilitate homologous recombination repair. *Nucleic Acids Res*, 40(2):682–91, 2012.
- [180] Z. Shu, S. Vijayakumar, C.-F. Chen, P.-L. Chen, and W.-H. Lee. Purified human SUV3p exhibits multiple-substrate unwinding activity upon conformational change. *Biochemistry*, 43(16):4781–4790, 2004.
- [181] B. L. Sibanda, D. Y. Chirgadze, and T. L. Blundell. Crystal structure of DNA-PKcs reveals a large open-ring cradle comprised of HEAT repeats. *Nature*, 463(7277):118–121, 2010.
- [182] M. Ślabicki, M. Theis, D. B. Krastev, S. Samsonov, E. Mundwiller, M. Junqueira, M. Paszkowski-Rogacz, J. Teyra, A. K. Heninger, I. Poser, F. Prieur, J. Truchetto, C. Confavreux, C. Marelli, A. Durr, J. P. Camdessanche, A. Brice, A. Shevchenko, M. T. Pisabarro, G. Stevanin, and F. Buchholz. A genome-scale DNA repair RNAi screen identifies SPG48 as a novel gene associated with hereditary spastic paraplegia. *PLoS Biol*, 8(6):e1000408, 2010.
- [183] A. Smogorzewska, R. Desetty, T. T. Saito, M. Schlabach, F. P. Lach, M. E. Sowa, A. B. Clark, T. A. Kunkel, J. W. Harper, M. P. Colaioco,

- and S. J. Elledge. A genetic screen identifies FAN1, a Fanconi anemia-associated nuclease necessary for DNA interstrand crosslink repair. *Mol Cell*, 39(1):36–47, 2010.
- [184] C. S. Sørensen, L. T. Hansen, J. Dziegielewska, R. G. Syljosen, C. Lundin, J. Bartek, and T. Helleday. The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair. *Nat Cell Biol*, 7(2):195–201, 2005.
- [185] C. Spycher, E. S. Miller, K. Townsend, L. Pavic, N. A. Morrice, P. Jancsak, G. S. Stewart, and M. Stucki. Constitutive phosphorylation of MDC1 physically links the MRE11RAD50NBS1 complex to damaged chromatin. *J Cell Biol*, 181(2):227–240, 2008.
- [186] J. M. Sterner, Y. Murata, H. G. Kim, S. B. Kennett, D. J. Templeton, and J. M. Horowitz. Detection of a novel cell cycle-regulated kinase activity that associates with the amino terminus of the retinoblastoma protein in G2/M phases. *J Biol Chem*, 270(16):9281–8, 1995.
- [187] G. S. Stewart, R. S. Maser, T. Stankovic, D. A. Bressan, M. I. Kaplan, N. G. Jaspers, A. Raams, P. J. Byrd, J. H. Petrini, and A. M. Taylor. The DNA double-strand break repair gene hMRE11 is mutated in individuals with an ataxia-telangiectasia-like disorder. *Cell*, 99(6):577–87, 1999.
- [188] G. S. Stewart, S. Panier, K. Townsend, A. K. Al-Hakim, N. K. Kolas, E. S. Miller, S. Nakada, J. Ylanko, S. Olivarius, M. Mendez,

- C. Oldreive, J. Wildenhain, A. Tagliaferro, L. Pelletier, N. Taubenheim, A. Durandy, P. J. Byrd, T. Stankovic, A. M. R. Taylor, and D. Durocher. The RIDDLE syndrome protein mediates a ubiquitin-dependent signaling cascade at sites of DNA damage. *Cell*, 136(3):420–434, 2009.
- [189] T. Stiff, M. O’Driscoll, N. Rief, K. Iwabuchi, M. Löbrich, and P. A. Jeggo. ATM and DNA-PK function redundantly to phosphorylate H2AX after exposure to ionizing radiation. *Cancer Res*, 64(7):2390–2396, 2004.
- [190] C. Stoepker, K. Hain, B. Schuster, Y. Hilhorst-Hofstee, M. A. Rooimans, J. Steltenpool, A. B. Oostra, K. Eirich, E. T. Korthof, A. W. Nieuwint, N. G. Jaspers, T. Bettecken, H. Joenje, D. Schindler, J. Rouse, and J. P. de Winter. SLX4, a coordinator of structure-specific endonucleases, is mutated in a new Fanconi anemia subtype. *Nat Genet*, 43(2):138–41, 2011.
- [191] D. Strumberg, A. A. Pilon, M. Smith, R. Hickey, L. Malkas, and Y. Pommier. Conversion of topoisomerase I cleavage complexes on the leading strand of ribosomal DNA into 5’-phosphorylated DNA double-strand breaks by replication runoff. *Mol Cell Biol*, 20(11):3977–87, 2000.
- [192] M. Stucki, J. A. Clapperton, D. Mohammad, M. B. Yaffe, S. J. Smerdon, and S. P. Jackson. MDC1 directly binds phosphorylated histone

- H2AX to regulate cellular responses to DNA double-strand breaks. *Cell*, 123(7):1213–1226, 2005.
- [193] A. N. Suhasini, N. A. Rawtani, Y. Wu, J. A. Sommers, S. Sharma, G. Mosedale, P. S. North, S. B. Cantor, I. D. Hickson, and R. M. Brosh, Jr. Interaction between the helicases genetically linked to Fanconi anemia group J and Bloom’s syndrome. *EMBO J*, 30(4):692–705, 2011.
- [194] K. Suzuki, S. Kodama, and M. Watanabe. Recruitment of ATM protein to double strand DNA irradiated with ionizing radiation. *J Biol Chem*, 274(36):25571–5, 1999.
- [195] J. M. Svendsen and J. W. Harper. GEN1/Yen1 and the SLX4 complex: Solutions to the problem of holliday junction resolution. *Genes Dev*, 24(6):521–36, 2010.
- [196] J. M. Svendsen, A. Smogorzewska, M. E. Sowa, B. C. O’Connell, S. P. Gygi, S. J. Elledge, and J. W. Harper. Mammalian BTBD12/SLX4 assembles a holliday junction resolvase and is required for DNA repair. *Cell*, 138(1):63–77, 2009.
- [197] J. A. Swenberg, K. Lu, B. C. Moeller, L. Gao, P. B. Upton, J. Nakamura, and T. B. Starr. Endogenous versus exogenous DNA adducts: Their role in carcinogenesis, epidemiology, and risk assessment. *Toxicol Sci*, 120(suppl 1):S130–S145, 2011.

- [198] E. M. Swisher, W. Sakai, B. Y. Karlan, K. Wurz, N. Urban, and T. Taniguchi. Secondary BRCA1 mutations in BRCA1-mutated ovarian carcinomas with platinum resistance. *Cancer Res*, 68(8):2581–6, 2008.
- [199] L. S. Symington and W. D. Heyer. Some disassembly required: role of DNA translocases in the disruption of recombination intermediates and dead-end complexes. *Genes Dev*, 20(18):2479–86, 2006.
- [200] R. J. Szczesny, H. Obriot, A. Paczkowska, R. Jedrzejczak, A. Dmochowska, E. Bartnik, P. Formstecher, R. Polakowska, and P. P. Stepień. Down-regulation of human RNA/DNA helicase SUV3 induces apoptosis by a caspase- and AIF-dependent pathway. *Biology of the Cell*, 099(6):323–332, 2007.
- [201] J. Tan, S. Cang, Y. Ma, R. L. Petrillo, and D. Liu. Novel histone deacetylase inhibitors in clinical trials as anti-cancer agents. *J Hematol Oncol*, 3:5, 2010.
- [202] T. Taniguchi, M. Tischkowitz, N. Ameiziane, S. V. Hodgson, C. G. Mathew, H. Joenje, S. C. Mok, and A. D. D’Andrea. Disruption of the Fanconi anemia-BRCA pathway in cisplatin-sensitive ovarian tumors. *Nat Med*, 9(5):568–74, 2003.
- [203] S. V. Tavtigian, J. Simard, J. Rommens, F. Couch, D. Shattuck-Eidens, S. Neuhausen, S. Merajver, S. Thorlacius, K. Offit, D. Stoppa-Lyonnet, C. Belanger, R. Bell, S. Berry, R. Bogden, Q. Chen,

- T. Davis, M. Dumont, C. Frye, T. Hattier, S. Jammulapati, T. Jannecki, P. Jiang, R. Kehrer, J. F. Leblanc, J. T. Mitchell, J. McArthur-Morrison, K. Nguyen, Y. Peng, C. Samson, M. Schroeder, S. C. Snyder, L. Steele, M. Stringfellow, C. Stroup, B. Swedlund, J. Swense, D. Teng, A. Thomas, T. Tran, M. Tranchant, J. Weaver-Feldhaus, A. K. Wong, H. Shizuya, J. E. Eyfjord, L. Cannon-Albright, F. Labrie, M. H. Skolnick, B. Weber, A. Kamb, and D. E. Goldgar. The complete BRCA2 gene and mutations in chromosome 13q-linked kindreds. *Nat Genet*, 12(3):333–7, 1996.
- [204] T. Thorslund, M. J. McIlwraith, S. A. Compton, S. Lekomtsev, M. Petronczki, J. D. Griffith, and S. C. West. The breast cancer tumor suppressor BRCA2 promotes the specific targeting of RAD51 to single-stranded DNA. *Nat Struct Mol Biol*, 17(10):1263–5, 2010.
- [205] O. Vafa, M. Wade, S. Kern, M. Beeche, T. K. Pandita, G. M. Hampton, and G. M. Wahl. c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability. *Mol Cell*, 9(5):1031–44, 2002.
- [206] A. J. van Gool, R. Verhage, S. M. Swagemakers, P. van de Putte, J. Brouwer, C. Troelstra, D. Bootsma, and J. H. Hoeijmakers. RAD26, the functional *s. cerevisiae* homolog of the Cockayne syndrome B gene ERCC6. *EMBO J*, 13(22):5361–9, 1994.

- [207] R. Varon, C. Vissinga, M. Platzer, K. M. Cerosaletti, K. H. Chrzanowska, K. Saar, G. Beckmann, E. Seemanov^o, P. R. Cooper, N. J. Nowak, M. Stumm, C. M. Weemaes, R. A. Gatti, R. K. Wilson, M. Digweed, A. Rosenthal, K. Sperling, P. Concannon, and A. Reis. Nibrin, a novel DNA double-strand break repair protein, is mutated in Nijmegen breakage syndrome. *Cell*, 93(3):467–76, 1998.
- [208] G. Vauquelin and S. J. Charlton. Long-lasting target binding and re-binding as mechanisms to prolong in vivo drug action. *Br J Pharmacol*, 161(3):488–508, 2010.
- [209] F. Vaz, H. Hanenberg, B. Schuster, K. Barker, C. Wiek, V. Erven, K. Neveling, D. Endt, I. Kesterton, F. Autore, F. Fraternali, M. Freund, L. Hartmann, D. Grimwade, R. G. Roberts, H. Schaal, S. Mohammed, N. Rahman, D. Schindler, and C. G. Mathew. Mutation of the RAD51C gene in a fanconi anemia-like disorder. *Nat Genet*, 42(5):406–9, 2010.
- [210] S. Venoe, T. Kulikowicz, C. Pestana, P. Stepień, T. Stevnsner, and V. Bohr. The human Suv3 helicase interacts with replication protein A and flap endonuclease 1 in the nucleus. *Biochem J*, 440(2):293–300, 2011.
- [211] J. R. Walker, R. A. Corpina, and J. Goldberg. Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. *Nature*, 412(6847):607–614, 2001.

- [212] R. Waltes, R. Kalb, M. Gatei, A. W. Kijas, M. Stumm, A. Sobeck, B. Wieland, R. Varon, Y. Lerenthal, M. F. Lavin, D. Schindler, and T. Drk. Human RAD50 deficiency in a Nijmegen breakage syndrome-like disorder. *Am J Hum Genet*, 84(5):605–16, 2009.
- [213] P. Watcharasit, G. N. Bijur, J. W. Zmijewski, L. Song, A. Zmijewska, X. Chen, G. V. W. Johnson, and R. S. Jope. Direct, activating interaction between glycogen synthase kinase-3 β and p53 after DNA damage. *Proc Natl Acad Sci U S A*, 99(12):7951–5, 2002.
- [214] T. Wechsler, S. Newman, and S. C. West. Aberrant chromosome morphology in human cells defective for Holliday junction resolution. *Nature*, 471(7340):642–6, 2011.
- [215] J. Wesoly, S. Agarwal, S. Sigurdsson, W. Bussen, S. Van Komen, J. Qin, H. van Steeg, J. van Benthem, E. Wassenaar, W. M. Baarends, M. Ghazvini, A. A. Tafel, H. Heath, N. Galjart, J. Essers, J. A. Grootegoed, N. Arnheim, O. Bezzubova, J. M. Buerstedde, P. Sung, and R. Kanaar. Differential contributions of mammalian Rad54 paralogs to recombination, DNA damage repair, and meiosis. *Mol Cell Biol*, 26(3):976–89, 2006.
- [216] R. S. Williams, G. E. Dodson, O. Limbo, Y. Yamada, J. S. Williams, G. Guenther, S. Classen, J. N. Glover, H. Iwasaki, P. Russell, and J. A. Tainer. Nbs1 flexibly tethers Ctp1 and Mre11-Rad50 to coordinate

- DNA double-strand break processing and repair. *Cell*, 139(1):87–99, 2009.
- [217] R. S. Williams, G. Moncalian, J. S. Williams, Y. Yamada, O. Limbo, D. S. Shin, L. M. Grocock, D. Cahill, C. Hitomi, G. Guenther, D. Moiani, J. P. Carney, P. Russell, and J. A. Tainer. Mre11 dimers coordinate DNA end bridging and nuclease processing in double-strand-break repair. *Cell*, 135(1):97–109, 2008.
- [218] A. K. Wong, P. A. Ormonde, R. Pero, Y. Chen, L. Lian, G. Salada, S. Berry, Q. Lawrence, P. Dayananth, P. Ha, S. V. Tavtigian, D. H. Teng, and P. L. Bartel. Characterization of a carboxy-terminal BRCA1 interacting protein. *Oncogene*, 17(18):2279–85, 1998.
- [219] A. K. Wong, R. Pero, P. A. Ormonde, S. V. Tavtigian, and P. L. Bartel. RAD51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene *brca2*. *J Biol Chem*, 272(51):31941–4, 1997.
- [220] R. Wooster, G. Bignell, J. Lancaster, S. Swift, S. Seal, J. Mangion, N. Collins, S. Gregory, C. Gumbs, and G. Micklem. Identification of the breast cancer susceptibility gene BRCA2. *Nature*, 378(6559):789–92, 1995.
- [221] L. Wu and I. D. Hickson. The Bloom’s syndrome helicase suppresses crossing over during homologous recombination. *Nature*, 426(6968):870–4, 2003.

- [222] W. Wu, K. Sato, A. Koike, H. Nishikawa, H. Koizumi, A. R. Venkitaraman, and T. Ohta. HERC2 is an E3 ligase that targets BRCA1 for degradation. *Cancer Res*, 70(15):6384–6392, 2010.
- [223] B. Xia, Q. Sheng, K. Nakanishi, A. Ohashi, J. Wu, N. Christ, X. Liu, M. Jasin, F. J. Couch, and D. M. Livingston. Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2. *Mol Cell*, 22(6):719–29, 2006.
- [224] D. Yang, F. Buchholz, Z. Huang, A. Goga, C. Y. Chen, F. M. Brodsky, and J. M. Bishop. Short RNA duplexes produced by hydrolysis with *Escherichia coli* RNase III mediate effective RNA interference in mammalian cells. *Proc Natl Acad Sci U S A*, 99(15):9942–7, 2002.
- [225] Y. Yonetani, H. Hocheegger, E. Sonoda, S. Shinya, H. Yoshikawa, S. Takeda, and M. Yamazoe. Differential and collaborative actions of Rad51 paralog proteins in cellular response to DNA damage. *Nucleic Acids Res*, 33(14):4544–52, 2005.
- [226] V. P. Yu, M. Koehler, C. Steinlein, M. Schmid, L. A. Hanakahi, A. J. van Gool, S. C. West, and A. R. Venkitaraman. Gross chromosomal rearrangements and genetic exchange between nonhomologous chromosomes following BRCA2 inactivation. *Genes Dev*, 14(11):1400–6, 2000.
- [227] X. Yu and R. Baer. Nuclear localization and cell cycle-specific expression of CtIP, a protein that associates with the BRCA1 tumor suppressor. *J Biol Chem*, 275(24):18541–9, 2000.

- [228] X. Yu, S. Fu, M. Lai, R. Baer, and J. Chen. BRCA1 ubiquitinates its phosphorylation-dependent binding partner CtIP. *Genes Dev*, 20(13):1721–6, 2006.
- [229] M. H. Yun and K. Hiom. CtIP-BRCA1 modulates the choice of DNA double-strand-break repair pathway throughout the cell cycle. *Nature*, 459(7245):460–3, 2009.
- [230] X. Zhao and G. Blobel. A SUMO ligase is part of a nuclear multiprotein complex that affects DNA repair and chromosomal organization. *Proc Natl Acad Sci U S A*, 102(13):4777–4782, 2005.
- [231] B. B. Zhou and J. Bartek. Targeting the checkpoint kinases: chemosensitization versus chemoprotection. *Nat Rev Cancer*, 4(3):216–25, 2004.
- [232] Q. Zhu, G. M. Pao, A. M. Huynh, H. Suh, N. Tonnu, P. M. Nederlof, F. H. Gage, and I. M. Verma. BRCA1 tumour suppression occurs via heterochromatin-mediated silencing. *Nature*, 477(7363):179–84, 2011.
- [233] L. Zou and S. J. Elledge. Sensing dna damage through ATRIP recognition of RPA-ssDNA complexes. *Science*, 300(5625):1542–8, 2003.