Review

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Shieldin – the protector of DNA ends

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Abstract

DNA double-strand breaks are a threat to genome integrity and cell viability. The nucleolytic processing of broken DNA ends plays a central role in dictating the repair processes that will mend these lesions. Usually, DNA end resection promotes repair by homologous recombination, whereas minimally processed ends are repaired by non-homologous end joining. Important in this process is the chromatin-binding protein 53BP1, which inhibits DNA end resection. How 53BP1 shields DNA ends from nucleases has been an enduring mystery. The recent discovery of shieldin, a four-subunit protein complex with single-stranded DNA-binding activity, illuminated a strong candidate for the ultimate effector of 53BP1-dependent end protection. Shieldin consists of REV7, a known 53BP1-pathway component, and three hitherto uncharacterized proteins: C20orf196 (SHLD1), FAM35A (SHLD2), and CTC-534A2.2 (SHLD3). Shieldin promotes many 53BP1-associated activities, such as the protection of DNA ends, non-homologous end joining, and immunoglobulin class switching. This review summarizes the identification of shieldin and the various models of shieldin action and highlights some outstanding questions requiring answers to gain a full molecular understanding of shieldin function.

Keywords DNA repair; end resection; genome stability; homologous recombination; non-homologous end joining

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See the Glossary for abbreviations used in this article.

Introduction

53BP1 is a chromatin-binding protein [1] that regulates DNA repair primarily by limiting long-range 5′–3′ nucleolytic digestion of DNA ends, a process known as DNA end resection [2]. The protection of DNA ends by the 53BP1-dependent pathway promotes physiological or pathological DNA double-strand break (DSB) repair by non-homologous end joining (NHEJ) despite the fact that 53BP1 is not a core component of the NHEJ machinery [3]. Indeed, 53BP1 is crucial for NHEJ-driven biological processes such as immunoglobulin class switching [4,5], the fusion of dysfunctional telomeres [6], and the chromosome aberrations caused by the exposure of *BRCA1*-deficient cells to poly(ADP-ribose) polymerase inhibitors (PARPi) [2] (Fig 1).

Intriguingly, 53BP1 is not necessary for all NHEJ-dependent repair reactions. Indeed, 53BP1 is involved in only a subset of V(D)J recombination events [4,5,7] and analysis of isogenic DT40 cell knockouts indicated that 53BP1 loss causes milder radiosensitization than mutations in the core NHEJ factor Ku70 [3]. Conversely, the ability of 53BP1 to limit the formation of single-stranded (ss) DNA at broken ends is not solely involved in regulating NHEJ. As an example, during the phases of the cell cycle where homologous recombination (HR) is active [8], 53BP1 influences the type of HR pathway used by modulating end resection [9]. Recent work indicates that 53BP1 also shields nascent DNA from degradation at stalled replication forks (Fig 1) [10,11]. 53BP1 is also active at dysfunctional telomeres that have been depleted of shelterin complex subunits [6,12]. TRF2-depleted telomeres undergo 53BP1- and NHEJ-dependent fusion, accentuating the role of 53BP1 as an NHEJ factor [6]. In contrast, 53BP1 prevents resection at TPP1-depleted telomeres without promoting NHEJ-driven fusion, suggesting a DNA end protection role independent of NHEJ [12]. Therefore, an emerging view of 53BP1 points to a role as a resection antagonist rather than a dedicated NHEJ factor.

Remarkably, the loss of 53BP1 reverses the cell and organismal lethality associated with mutations in *BRCA1* [2,13,14], and loss-of-function mutations in *53BP1* lead to PARPi resistance in both cell and pre-clinical mouse tumor models of BRCA1 deficiency [15,16]. Loss of 53BP1 in BRCA1-deficient cells restores, to some degree, homologous recombination in a manner that depends on the activation of end resection [2]. This extraordinary genetic interaction points to a unique antagonism between BRCA1 and 53BP1, a conclusion supported by cell biological studies where BRCA1 and 53BP1 appear to compete for accumulation at DNA damage sites [17–20]. These findings suggest that initiating end resection is a key decision point in DSB repair pathway choice, with a direct impact on the therapeutic efficacy of PARP inhibitors.

How 53BP1 impacts DNA repair has long been enigmatic, but it is certain that its action requires its recruitment to DSB sites [21–23]. 53BP1 accumulates on the chromatin surrounding DSB sites by recognizing dually modified nucleosomes containing histone H4 methylated on its Lys20 residue and histone H2A ubiquitylated on Lys15 [21,24]. Since H4K20 methylation is nearly ubiquitous, H2AK15 ubiquitylation by RNF168 provides the first DNA damage-dependent signal leading to 53BP1 recruitment. 53BP1 must be at

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Glossary

53BP1	p53-binding protein 1
AP	affinity purification
APEX2	ascorbate peroxidase 2
ATM	ataxia telangiectasia mutated
ATMIN	ataxia telangiectasia-mutated interactor
BirA	bifunctional ligase/repressor A
BLM	Bloom syndrome protein
BRCA1	breast cancer type 1 susceptibility protein
BrdU	bromodeoxyuridine
C20orf196	chromosome 20 open reading frame 196
CDC20	cell division cycle 20
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats/CRISPR-associated 9
CSR	class switch recombination
CST	CTC1-STN1-TEN1
CTC1	conserved telomere maintenance component 1
CtIP	CtBP-interacting protein
DNA2	DNA replication helicase/nuclease 2
DNA	deoxyribonucleic acid
DSB	double-strand break
DT40	avian leukosis virus-induced bursal lymphoma cell line derived from a Hyline SC chicken
DYNLL1	dynein light chain LC8-type 1
eIF4E	eukaryotic translation initiation factor 4E
EXO1	exonuclease 1
FAM35A	family with sequence similarity 35, member A
FHA	forkhead-associated
GFP	green fluorescent protein
H2AK15	histone H2A lysine 15
H4K20	histone H4 lysine 20
HEK293T	human embryonic kidney 293 cells containing the SV40 T-antigen.
HORMA	HOP1, REV7, MAD2
HR	homologous recombination
ICL	interstrand crosslinking
IR	ionizing radiation
Ku70	Ku autoantigen, 70 kDa
MAD2	mitotic arrest deficient 2
MDC1	mediator of DNA damage checkpoint 1
MMC	mitomycin C
MRE11	meiotic recombination 11
mRNA	messenger RNA
MRN	MRE11-RAD50-NBS1
MS	mass spectrometry
NHEJ	non-homologous end joining
nM	nanomolar
nt	nucleotides
OB-fold	oligosaccharide/oligonucleotide binding fold
PARPi	Poly(ADP-ribose) polymerase inhibitor
Pol α-primase	DNA polymerase alpha-primase
PTIP	PAX-interacting protein 1
RAD51	radiation sensitive 51
RBM	REV7 binding motif
REV7/MAD2L2	revertibility protein 7/mitotic arrest deficient 2-like protein 2
RIF1	RAP1-interacting factor 1
RINN	REV7-interacting novel NHEJ regulator
RNF168	RING finger protein 168
RNF8	RING finger protein 8
RPA1	replication protein A 70 kDa DNA-binding subunit
RPA	replication protein A
SHLD	shieldin
ssDNA	single-stranded DNA
TIRR	Tudor-interacting repair regulator protein
TLS	translesion DNA synthesis
TPP1	TINT1/PTOP/PIP1
TRAPPC13	trafficking protein particle complex subunit 13
TRF2	telomeric repeat-binding factor 2
TRIP13	thyroid hormone receptor interactor 13
V(D)J	variable, diversity, and joining gene segment

minimum a dimer to accrue on the chromatin flanking DSBs [21,25], leading to a model where the 53BP1–nucleosome interaction enhances the ability of chromatin to inhibit DNA end resection [26].

However, the function of 53BP1 in DNA repair also requires interacting partners, indicating that its interaction with nucleosomes alone is not sufficient to block DNA end processing. 53BP1 is phosphorylated by ATM on over 25 sites concentrated in the N-terminal half of the protein [27,28]. 53BP1 phosphorylation provides a second DNA damage-induced signal leading to the activation of the DNA repair function of 53BP1 [22,23] and promotes its interaction with two proteins, PTIP [29] and RIF1 [18–20,30,31]. These two proteins are involved in limiting end resection at DSBs independently of each other [32]. How RIF1 and PTIP collaborate to mediate 53BP1-dependent DNA repair is not understood and is likely complex [33], but genetic studies suggest that it is RIF1, not PTIP, that promotes the function of 53BP1 in many NHEJ-driven processes such as immunoglobulin class switching [32,33].

In 2015, two reports identified the small HORMA domain-containing protein REV7/MAD2L2 as a factor acting downstream of 53BP1 and RIF1 [34,35]. Indeed, REV7 is critical for mediating the cytotoxic effects of PARPi in *BRCA1*-deficient cells, immunoglobulin class switching, and fusion of dysfunctional telomeres [34,35]. The robust genetic data linking REV7 to 53BP1-dependent DNA repair was as convincing as it was confusing: How can this small protein, better known for its function in translesion DNA synthesis (TLS) as part of DNA polymerase ζ [36], antagonize DNA end resection? These findings raised the distinct possibility that additional 53BP1 effectors remained to be identified. In retrospect, this possibility clearly resonated with many in the field since (unbeknownst to most) a race for the identification for new factors involved in 53BP1-dependent DNA repair had just begun.

The hunt for the missing effectors of 53BP1

The search for the elusive 53BP1 effectors utilized various strategies that remarkably all converged on the same protein complex. One strategy involved CRISPR/Cas9-based pooled genetic screens to identify factors whose mutation conferred resistance to PARPi in *BRCA1*-mutated cells. These screens took advantage of the ability of *53BP1* mutations to suppress the sensitivity of *BRCA1*-mutated cells to PARPi, reasoning that mutation of 53BP1 effectors should do the same [37,38]. Noordermeer *et al* additionally mined a screen aimed at finding genes that promote resistance to ionizing radiation (IR), which is mediated in large part by NHEJ-dependent DNA repair [37]. These screens identified the previously uncharacterized proteins C20orf196 and FAM35A as promoters of NHEJ and suppressors of HR. Additionally, Noordermeer *et al* also identified CTC-534A2.2 as a factor acting alongside C20orf196 and FAM35A. CTC-534A2.2 is a protein encoded by an alternative transcript emanating from the *TRAPPC13* locus and was not annotated in

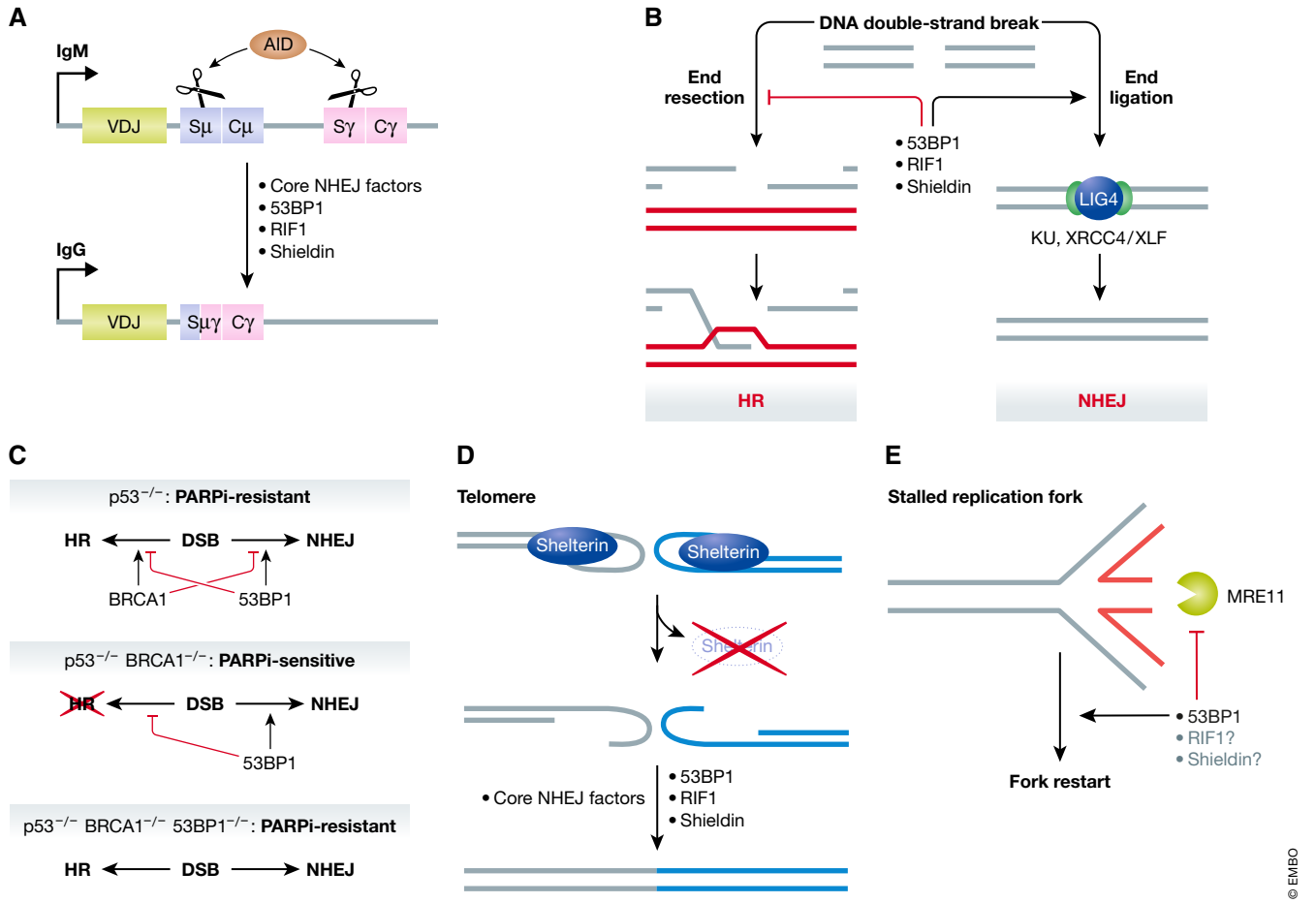

Figure 1. 53BP1 and shieldin act in various physiological contexts.
(A) 53BP1, RIF1, and shieldin mediate immunoglobulin class switch recombination. During B-cell stimulation, the activation-induced cytidine deaminase (AID) enzyme causes single-stranded breaks at two switch regions within the immunoglobulin heavy chain locus. 53BP1, RIF1, and shieldin are essential for non-homologous end joining (NHEJ)-mediated fusion of the two distant switch regions, altering the antibody subtype expressed from the locus. (B) Genomic DNA double-strand breaks can be repaired through two competing pathways: the resection-dependent homologous recombination or resection-independent direct ligation through NHEJ. The components of the 53BP1 pathway inhibit end resection and facilitate repair via NHEJ. (C) 53BP1 and BRCA1 antagonize each other. In a *Δp53* background, BRCA1 promotes HR and inhibits NHEJ while 53BP1 promotes NHEJ and inhibits HR, resulting in both DSB repair pathways being active. HR-proficient cells are resistant to PARP inhibition (PARPi). In the absence of BRCA1, 53BP1 inhibits HR, resulting in HR deficiency and PARPi sensitivity. Concurrent depletion of BRCA1 and 53BP1 results in the de-repression of HR, resulting in PARPi resistance. BRCA1 depletion is lethal in p53-proficient cells unless accompanied by a depletion of 53BP1. (D) Telomere dysfunction due to shelterin subunit depletion results in aberrant DNA end processing. TRF2 depletion results in 53BP1-dependent fusion of telomeres. (E) 53BP1 prevents MRE11-mediated degradation of stalled replication forks. During DNA replication, stalled replication forks can reverse into the "chicken-foot" configuration depicted. The nascent DNA portion (red) is a substrate for MRE11-mediated exonucleolytic degradation. 53BP1 prevents this degradation and promotes fork restart, while the involvement of RIF1 and shieldin in this context has not been characterized.

many databases, which explains why it was identified only by a subset of the groups who were searching for 53BP1 effectors. These three hitherto uncharacterized proteins arose in vertebrates, with the complete set co-occurring in species that perform immunoglobulin class switching [39].

As alternative approaches, all groups either based their searches on proximity labeling or affinity purification (AP) mass spectrometry (MS), or complemented their genetic screens with MS-based approaches. In particular, fusions of 53BP1 or REV7 with either the APEX2 peroxidase [39] or a promiscuous form of the BirA biotin ligase [40], respectively, allowed for selective biotinylation of proteins in close proximity to these baits in cells. AP-MS was also used to identify proteins interacting with REV7 [37,38,40–42], or, after their initial identification, partners of FAM35A, C20orf196, and CTC-534A2.2 [37,39,42]. In a more targeted approach, interaction partners of REV7 mutants specifically defective in CSR were also identified by AP-MS [43].

C20orf196, FAM35A, CTC-534A2.2, and REV7 form a stable complex even in the absence of exogenous DNA damage [37–40,43]. This protein complex was named "shieldin", a term originally coined a few years ago by Jiri Lukas to describe the idea that 53BP1 protects DNA ends in a manner analogous to the telomere end-protecting complex shelterin [44]. The previously uncharacterized components of shieldin were also given new names, using either the SHLD (shieldin) or the alternate RINN (REV7-interacting novel NHEJ regulator) nomenclature: C20orf196 was renamed

SHLD1/RINN3; FAM35A, SHLD2/RINN2; and CTC-534A2.2, SHLD3/RINN1. For the sake of clarity, we will employ the SHLD1/2/3 nomenclature for the remainder of this review.

Shieldin promotes PARP inhibitor cytotoxicity in BRCA1-mutated cells

Studies assessing depletion of the newly identified shieldin subunits revealed pronounced and highly consistent phenotypes. In particular, its role in antagonizing homologous recombination was validated exhaustively. Depleting any single subunit in various BRCA1-deficient cell lines suppressed their sensitivity to PARPi to a degree comparable to that of 53BP1 or REV7 depletion [37–39,43,45]. The potency of this effect was illustrated *in vivo* in mouse allograft experiments where *Brca1*-null mammary tumor cells edited to mutate *SHLD1* or *SHLD2* prior to allografting were resistant to PARPi treatment [37]. In addition, expression levels of *SHLD1* and *SHLD2* correlated with PARPi sensitivity in patient-derived xenografts of *BRCA1*-null tumors [38]. These findings suggest that shieldin mutations modify PARPi responses in *BRCA1*-mutated tumors.

CRISPR-mediated knockout of *Shld1* or *Shld2* enabled p53-proficient mouse embryonic stem cells to survive *Brca1* loss [37], recapitulating the profound genetic interaction observed between 53BP1 and BRCA1 [2,13,14]. Whether or not shieldin gene mutations will suppress embryonic lethality caused by BRCA1 loss remains to be determined. Nevertheless, the concomitant loss of BRCA1 with any of the newly identified shieldin subunits restores HR as observed by gene conversion assays and RAD51 ionizing radiation-induced focus formation [37–40,42]. These phenotypes closely mirror those of 53BP1 or REV7 loss in the context of HR suppression [2,34], further suggesting that shieldin acts in the same pathway as 53BP1.

Shieldin promotes 53BP1-dependent NHEJ

The role of 53BP1 in promoting NHEJ in a variety of physiological and pathological contexts is also shared by shieldin. Shieldin loss confers sensitivity to IR, the DNA topoisomerase II inhibitor etoposide, and the radiomimetic drug bleomycin [37–40,42], as was seen previously with the loss of REV7 [35]. More direct NHEJ measurements via random plasmid integration and the EJ5-GFP reporter assay revealed that every shieldin subunit, including REV7, contributes to NHEJ [35,37–42]. Although 53BP1 is not a core NHEJ component, it is essential for the long-range fusion of deprotected telomeres in cells deficient for the shelterin complex subunit TRF2 [6]. This feature is shared with shieldin, where loss of any subunit reduces the fusion of deprotected telomeres [35,38,39].

Shieldin also participates in 53BP1-dependent immunoglobulin class switching (also known as class switch recombination or CSR). CSR involves the long-range end joining of two DSBs within the immunoglobulin heavy chain-coding gene, which generates a large deletion that alters the antibody subtype (Fig 1A) [46]. 53BP1 is essential for CSR [4,5] and loss of any shieldin component, including REV7, similarly impairs this process [34,35,37–40,43]. Of further importance to immune system development, 53BP1-deficient mice also have decreased numbers of B lymphocytes due to partially defective V(D)J recombination [7]. However, in a departure from perfectly phenocopying of 53BP1 loss, genetic ablation of REV7 did not affect B-cell numbers, suggesting that shieldin does not participate in this pathway [43].

Epistasis between the 53BP1-RIF1 axis and shieldin

The genetic evidence presented in these multiple contemporaneous studies paints a compelling picture of shieldin sharing the same functions as 53BP1, and multiple lines of evidence indicate that shieldin acts genetically as part of a 53BP1-RIF1-shieldin pathway. Shieldin genes are epistatic with *53BP1* or *RIF1* with respect to CSR [37], sensitivity to DSB-inducing drugs [42], and rescue of HR in *BRCA1*-mutated cells [37,38]. Shieldin components are also epistatic to each other, consistent with them being subunits of the same complex [37,38,42,43].

Several intriguing results deviate from perfect epistasis within this pathway. In one study, BRCA1/SHLD1 and BRCA1/SHLD2 double-knockout cells were observed to be much more sensitive to ionizing radiation than BRCA1/53BP1 knockout cells [38]. Different subunits of shieldin may also have slightly different roles. Knocking out *SHLD1* in DT40 cells has a modest but reproducible sensitivity to the topoisomerase I poison camptothecin, while a *SHLD2* knockout has no effect [42]. Additionally, one study found that *SHLD1* and *SHLD2* knockout cells are sensitive to the DNA interstrand crosslinking (ICL) agent cisplatin, a phenotype often observed in cells with defects in the TLS, Fanconi anemia, or HR pathways [38,47]. However, other reports show that *SHLD3* knockout does not affect response to ICL agents [43] and that REV7 involvement in DSB repair pathway choice is distinct from its role in TLS [34,35,43]. Whether these observations represent true mechanistic differences or clonal/experimental variation remains to be determined, but they raise the possibility that some of the newly characterized shieldin components might have functions outside the 53BP1-RIF1-shieldin pathway, just as REV7 does.

REV7 in the context of shieldin

Aside from its role as a subunit of shieldin, REV7 is best known as an integral member of the TLS polymerase Pol ζ complex, where it binds to REV1 and REV3L [48]. This complex responds to DNA damage caused by a variety of lesions but is particularly important for the tolerance of DNA interstrand crosslinks such as those caused by the drugs cisplatin or mitomycin C (MMC) [49,50]. Indeed, biallelic mutations in *REV7* were found in a patient displaying a Fanconi anemia-like syndrome, a disease characterized by interstrand crosslink sensitivity, highlighting the importance of Pol ζ for the tolerance of such lesions [51].

REV7 is a 211-amino acid residue protein consisting entirely of a HORMA domain (Fig 2A). Within Pol ζ, REV3L has two conserved REV7-binding motifs (RBMs) defined by a P-x-x-x-p-P motif (x represents any amino acid, uppercase P represents a proline residue essential for the interaction, while lowercase p represents a less important proline residue) [52–54]. Analysis of REV7 mutants by Ghezraoui *et al* revealed that the Y63A and W171A mutants are unable to bind REV3L and are inactive in TLS, as shown by their inability to rescue the sensitivity of *REV7*-null cells to MMC [43]. However, REV7-W171A, but not the Y63A variant, was able to rescue the CSR defect of a *REV7* deletion, consistent with the finding that REV7 has distinct contributions to TLS and DSB repair [34,35,43]. Importantly, the W171A mutant, but not the Y63A mutant, can bind SHLD3, providing a compelling rationale for the observed separation of function [43]. Two RBMs are found in the SHLD3 N-terminus; REV7 interacts with a fragment of SHLD3 (amino acids 28–83) that harbors an RBM necessary for REV7 binding [39,43]. SHLD3 interacts with RIF1 in

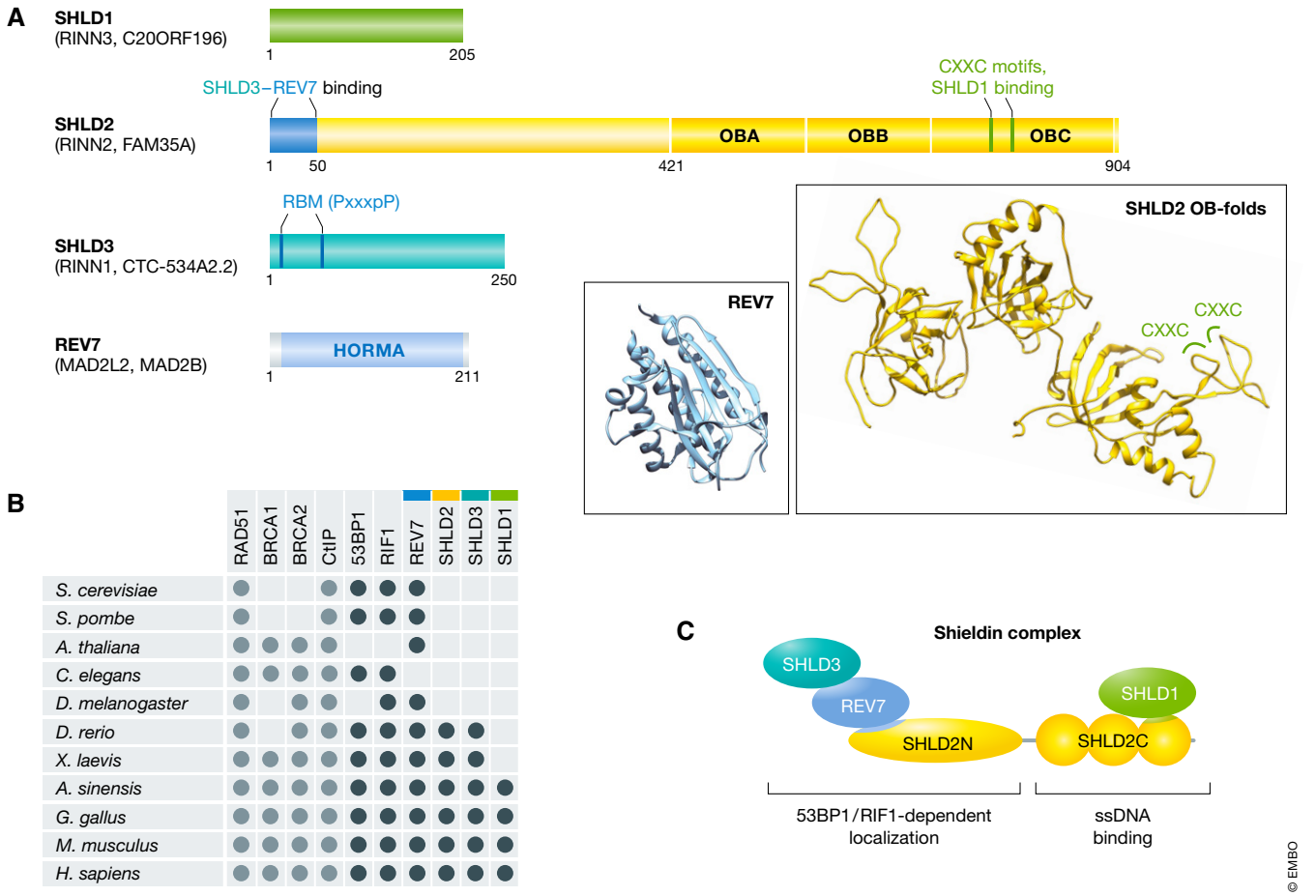

Figure 2. Schematic of shieldin subunits and the architecture of the complex.
(A) Amino acid residues of interest are numbered over each shieldin component. The three predicted tandem oligonucleotide/oligosaccharide-binding (OB) folds in SHLD2 and the HORMA domain of REV7 are depicted. SHLD2 contains a CXXC zinc-finger motif while SHLD3 has a REV7-binding motif (RBM). Within the RBM (PxxxpP), P is an essential proline, p denotes an optional proline, and x represents any amino acid. Ribbon structures: The predicted structure of the SHLD2 OB-folds (generated by homology modeling using the RPA1 structure; PDB:4GNX) with two CXXC zinc-finger motifs highlighted is shown in yellow. The structure of REV7 (PDB:3ABE) is shown in cyan. (B) Evolutionary conservation of shieldin and other proteins involved in DNA double-strand break repair pathway choice, based on known orthologues and BLAST homology search. (C) Functional architecture of the shieldin complex. SHLD3 and REV7 associate with the SHLD2 N-terminus, forming the 53BP1- and RIF1-dependent localization module. Meanwhile, SHLD1 associates with the SHLD2 C-terminus, forming the ssDNA-binding module.

co-immunoprecipitation experiments, while REV7 bridges SHLD3 to the rest of the complex by directly binding SHLD2 [37,39,43]. These findings suggest that SHLD3 and REV7 are the most 53BP1-RIF1-proximal elements within shieldin and form a localization module within the complex.

Like other HORMA-domain proteins, REV7 contains a stereotypical "safety belt" that encircles the domain's binding partners in its C-terminus [36]. This safety belt region of REV7 facilitates its interaction with SHLD3 [43]. In the case of the REV7 paralog MAD2, the safety belt is remodeled from a closed to an open conformation by the AAA^+ ATPase TRIP13 [55,56]. This remodeling into the open conformation ablates binding of MAD2 to its binding partner CDC20, modulating its function through conformational modification [55–57]. The idea that the REV7 safety belt may also be remodeled to modulate the 53BP1-RIF1-shieldin pathway is a tantalizing possibility that should be thoroughly investigated, especially since TRIP13 can be co-immunoprecipitated with REV7 [37].

Shieldin is a triple OB-fold-containing complex

Aside from REV7, none of the other shieldin components had been previously characterized. SHLD1 and SHLD3 are, like REV7, relatively small proteins of 205- and 250-amino acid residues in size, respectively, whereas SHLD2 is the largest subunit at 904 residues (Fig 2A). Shieldin homologues arose relatively late in evolution compared to other DSB repair pathway choice proteins, with conserved sequences found primarily in vertebrates (Fig 2B). Structure-based homology searches and predictions suggest that the C-terminal half of SHLD2 forms three tandem oligosaccharide/oligonucleotide binding (OB) folds [37,38,40,42], while a region of SHLD3 has limited homology to the mRNA cap-binding domain of the translation elongation initiation factor eIF4E [43].

The N-terminal half of SHLD2 is predicted to be disordered, with a conserved region in the first 100 residues. In particular, the first 50 residues of SHLD2 are sufficient and necessary for interaction with SHLD3 and REV7 [37,39]. Within this region of

SHLD2, two conserved prolines (P14 and P17) are essential for REV7 interaction [42]. The C-terminal half of SHLD2 is predicted at high confidence to form three tandem OB-folds similar to those found in RPA1 and CTC1 [37,38,40,42], the two largest subunits of the RPA (replication protein A) and CST (CTC1-STN1-TEN1) ssDNA-binding complexes, respectively. OB-folds are ssDNA-binding domains that are found in multiple proteins involved in genome stability [58]. One of the loops in the third OB-fold domain is predicted to form two CXXC-type zinc-finger motifs [38]. This putative zinc-finger-containing region is important for the association of SHLD2 with SHLD1 [38]. One report predicted that SHLD1 contains a winged helix domain in its C-terminus that is similar to the one present in the STN1 subunit of the CST complex [38]. The relatively large size of SHLD2 compared to that of the other shieldin subunits suggests that it serves as the core scaffold of the complex, and the presence of tandem OB-folds in its C-terminus may point toward a direct mechanism of action for shieldin through ssDNA binding.

Shieldin is recruited to DSB sites and represses resection

As expected of an effector of 53BP1, each subunit of shieldin, including REV7, accumulates at DSB sites [37–40,42] downstream of 53BP1 and RIF1 [37,38]. In agreement with the protein–protein interaction studies, assays of recruitment to DNA damage sites showed that SHLD3 is the subunit most proximal to RIF1, followed by REV7, SHLD2, and SHLD1 [37,38]. In fact, shieldin can be roughly divided into two modules: one module composed of SHLD3-REV7 and the N-terminal 50 residues of SHLD2 forming a "localization module", whereas the complex formed by SHLD1 and C-terminal OB-folds of SHLD2 form a ssDNA-binding module, as will be discussed in detail below (Fig 2B).

The decision point of DSB pathway choice revolves around end resection [8,59,60]. HR requires extensive degradation of the 5′ strand relative to the DSB, generating long tracts of ssDNA used for RAD51-mediated homology searching [61]. Initiation of resection in mammals occurs in a two-step process [62,63]: First, the MRE11-RAD50-NBS1 (MRN) resection complex induces endonuclease-generated nicks on the 5′-terminated strands on either side of the break with the aid of CtIP [64–66]. The resulting nick is then expanded through the 3′–5′ exonuclease activity of MRN and the 5′–3′ exonuclease activity of EXO1 or DNA2-BLM [67,68]. The resulting large tracts of ssDNA are bound by RPA, which is then replaced by RAD51 to initiate homology searching, strand invasion, and copying of homologous sequences.

Multiple lines of evidence indicate that shieldin antagonizes DNA end resection. First, depletion of shieldin subunits increases the levels of phosphorylated RPA after induction of DSBs [35,37,38,40], which is a surrogate readout for ssDNA formation [69]. Similarly, induction of DSBs in shieldin-depleted cells results in increased numbers of RPA foci measured by immunofluorescence [34,38,39]. Secondly, the levels of RPA bound at the immunoglobulin gene switch regions are increased following induction of class switching as determined by chromatin immunoprecipitation [34,43]. Thirdly, cells with shieldin gene knockouts display an increased amount of ssDNA after camptothecin treatment as measured by native BrdU labeling, also reflective of extensive DNA end resection [38,39]. Finally, measurement of resection by native Southern blotting at deprotected telomeres provides a direct assessment of end resection, and analysis of shieldin depletion using the Cre-mediated removal of *Tpp1* or *Trf2* showed that loss of shieldin increases the formation of ssDNA at deprotected telomeres [45]. Therefore, like 53BP1, shieldin opposes resection, but the key question remains whether it does so directly through an inherent activity of the complex or indirectly through the recruitment of other factors.

SHLD2 ssDNA-binding activity is important for shieldin function

The predicted presence of the OB-fold domains in the SHLD2 C-terminus provided the first clue into the biochemical activity of shieldin. Expression of a SHLD2 variant lacking its OB-folds fails to complement the IR sensitivity of *SHLD2*-knockout cells or restore PARPi sensitivity in the *SHLD2*/*BRCA1* double-knockout cells [38]. These findings suggest that the SHLD2 OB-folds are critical for the function of the complex.

If the OB-folds of SHLD2 are critical effectors of the 53BP1-RIF1 pathway, it then follows that a major role for 53BP1 in suppressing HR is the recruitment of SHLD2 to sites of DNA damage. To test this possibility, Noordermeer *et al* [37] artificially recruited SHLD2 to DSB sites via its fusion to the FHA domain of RNF8. The resulting FHA-SHLD2 fusion impaired RAD51 IR-induced focus formation in *BRCA1/53BP1*-double-knockout cells, consistent with an inhibition of HR [37]. Importantly, mutations designed to remove key ssDNA-interacting aromatic residues in the SHLD2 OB-folds resulted in a fusion protein that was unable to suppress HR. Furthermore, fusion of a truncated variant of SHLD2, consisting solely of the OB-folds, to the RNF8 FHA domain, was sufficient to fully recapitulate the inhibition of HR in *BRCA1/53BP1*-double-knockout cells [37]. A similar set of mutants assessed by Dev *et al* [38] were unable to complement the IR sensitivity of *SHLD2* knockout cells. Collectively, these results identify the predicted OB-fold domains of SHLD2 as critical elements of the 53BP1-RIF1-shieldin pathway.

Although structural information on the SHLD2 OB-folds is currently unavailable, the purified SHLD2 C-terminus binds to DNA, with a strong preference for ssDNA that is consistent with the binding properties of other OB-fold proteins [37,38,40,42]. ssDNA binding *in vitro* is abolished by the same aromatic residue mutations that disable the ability of SHLD2 to suppress HR, underlining ssDNA binding as a key function of SHLD2 [37,38]. In all cases, ssDNA binding was determined using ssDNA templates > 50 nt [37,38,40,42] and one report observed that the SHLD2 C-terminus cannot bind 30-nt substrates [42]. The requirement for long ssDNA substrates is surprising, as tandem OB-fold-containing proteins and complexes often reach their peak binding affinity at a substrate length of 35 nt or less [70–72].

Intriguingly, the affinity of SHLD2 for ssDNA appears highly variable dependent on context. The SHLD2 C-terminus purified in complex with SHLD1 from HEK293T cells binds ssDNA with a dissociation constant of approximately 10 nM [37], an intermediate affinity between RPA (< 1 nM) and RAD51 (> 100 nM) [71,73]. However, the SHLD2 C-terminus expressed in *Escherichia coli* has 1–2 orders of magnitude lower affinity for ssDNA than the protein complex purified from human cells [38,42]. Co-expression with SHLD1 increases the stability of the SHLD2 C-terminus in mammalian cells [37], but whether SHLD1 stimulates the affinity of SHLD2 for ssDNA remains an open question. Alternatively, there may either be mammalian-specific post-translational modifications that

increase the affinity of SHLD2 for ssDNA or additional components of shieldin that remain unidentified.

The shieldin paradox

The extensive genetic and biochemical characterization of shieldin presented above converges into one central paradox: Why would a complex that prevents end-resection function by binding to ssDNA? NHEJ has mechanisms for processing short overhangs [74], but ssDNA longer than 20–30 nt is characteristic of resection. If shieldin binds to ssDNA after the initiation of resection, how would the complex interrupt a processive nuclease acting upon its substrate? Resolving this paradox is key to understanding shieldin function and may reveal a key step in DSB repair pathway choice.

One possibility to solve this paradox is that shieldin promotes fill-in synthesis at resected ends rather than blocking end-resection nucleases per se. Indeed, shieldin interacts with the CST complex, which also antagonizes end resection [45]. During telomere replication, CST interacts with the polymerase alpha-primase complex (Pol α-primase) to synthesize DNA, filling in the excessively long overhangs of nascent telomeres [75]. CST-Polα is recruited to telomeres through the OB-fold-containing shelterin complex [75]. Analogously, Mirman *et al* found that CST is recruited to DSBs in a 53BP1- and shieldin-dependent manner, with knockdown of CST components promoting resection and suppressing the sensitivity of BRCA1-null cells to PARPi [45,76]. Furthermore, the suppression of PARPi sensitivity through CST knockdown is epistatic to either 53BP1 or REV7 knockout [45]. Radial chromosome formation in PARPi-treated *BRCA1*-null cells, a characteristic sign of PARPi toxicity, is suppressed by inhibition of Polα [45]. These findings provide a strong argument toward the involvement of CST-Polα in HR suppression through the 53BP1-RIF1-shieldin pathway.

Despite being a compelling mechanism of action to antagonize resection, the role of fill-in synthesis by CST-Polα also raises multiple questions with respect to the 53BP1-shieldin pathway. First, it is unclear how CST-Polα-mediated DNA synthesis and SHLD2-mediated ssDNA binding are integrated to modulate end resection. Second, as EXO1 and DNA2-BLM can generate long ssDNA tracts, the poor processivity of Pol α-primase [77] and its lack of proofreading activity [78] seems problematic unless more processive polymerases take over after synthesis is initiated. Regardless of these

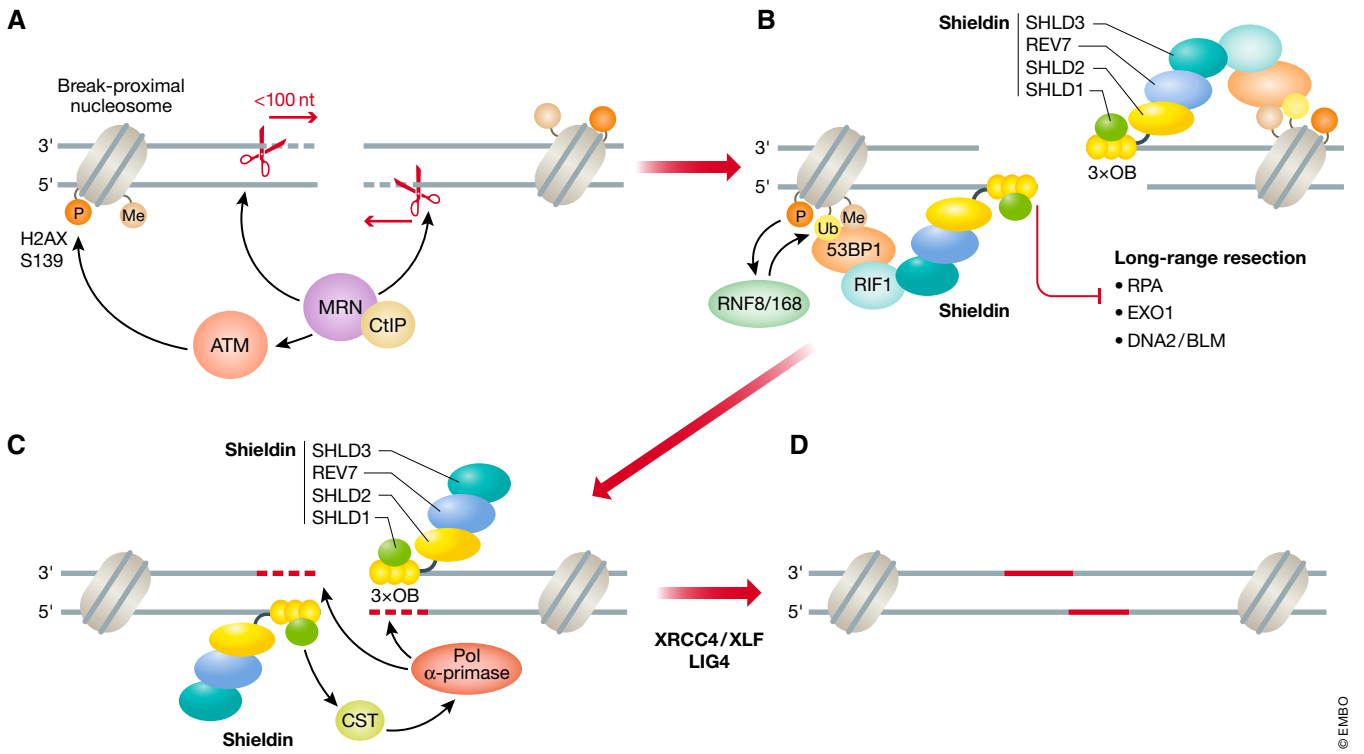

Figure 3. Proposed 53BP1-RIF1-shieldin mechanism of action in DNA double-strand break repair.
(A) The MRE11-RAD50-NBS1 (MRN) complex and its accessory factor CtIP is recruited to DNA double-strand break sites and introduces an endonuclease nick on the 5′-terminated strand, which is expanded toward the break via the 3′–5′ exonuclease activity of MRE11. The nucleolytic activity of MRN results in short (< 100 nucleotides) tracts of single-stranded (ss) DNA around the break. The NBS1 subunit of MRN also recruits and activates the ataxia telangiectasia-mutated (ATM) kinase that phosphorylates histone H2AX at serine 139 (γH2A.X) on nucleosomes surrounding the DSB. (B) γH2AX recruits the RNF8 ubiquitin ligase through MDC1 binding (not shown). RNF8 catalyzes the K63-linked polyubiquitination of histone H1, which in turn recruits RNF168. RNF168 ubiquitinates histone H2A lysine 15 which, in concert with constitutive H4 lysine 20 methylation, recruits 53BP1. 53BP1 recruits RIF1 in an ATM-dependent manner, which localizes the shieldin complex to the DSB. The three tandem OB-folds of SHLD2 binds the short ssDNA tract and inhibits long-range 5′–3′ resection mediated by EXO1 and DNA2/BLM nucleases. (C) Shieldin then recruits the CTC1-STN1-TEN1 (CST) complex and its binding partner, the polymerase α-primase (Pol α-primase) complex to the DSB site. Pol α-primase fills in the short ssDNA tract by synthesizing new DNA (red). (D) The DSB is subsequently repaired by non-homologous end joining via DNA ligase IV (LIG4) and its accessory factors XRCC4/XLF. P, Ub, and Me represent phosphorylated, ubiquitinated, and methylated histones, respectively. S1 represents SHLD1. Black dashed lines indicate short range resection, while red dashed lines indicate fill-in synthesis.

possibilities, we view the identification of shieldin mutants that are unable to interact with CST as an important first step in helping untangle the respective contributions of CST-Polα and SHLD2 ssDNA binding in this process.

Another means by which shieldin may antagonize resection is by inhibiting the nucleases involved in long-range resection. Resection at blocked DNA ends is initiated by the combined action of MRN and CtIP, which generates an endonuclease cleavage between 20 and 40 nucleotides away from the blockage, which can include nucleosomes or the DNA end-binding factor Ku (Fig 3A) [66,79]. This endonuclease cut is then extended toward the break using by the 3′–5′ exonuclease activity of MRE11 followed by the long-range 5′–3′ resection away from the break by EXO1 and DNA2/BLM (Fig 3A). The initial MRE11 processing generates ssDNA of similar length to the preferred shieldin substrate, and may provide an opportunity to interrupt the switch between short- to long-range resection, possibly through competition with RPA or steric occlusion of EXO1 and DNA2-BLM (Fig 3B). The MRE11-resected product is also short enough to be conceivably repaired by Pol α-primase (Fig 3C). After Polα fill-in of the ssDNA generated by MRN resection, the break could then be repaired through NHEJ (Fig 3D). Furthermore, the NHEJ-promoting Ku complex also protects DNA ends, but is removed by the MRE11-dependent resection initiation step [80]. Consistent with this model of Ku and shieldin acting on different steps of resection, their mutations are not epistatic to each other [42]. Since end resection can be recapitulated *in vitro* [81], it should be feasible to similarly reconstitute shieldin-dependent end protection.

Future perspectives

The discovery of shieldin is a new and exciting chapter in our understanding of the regulation of DSB repair. Until the identification of shieldin, there were very few hints as to the molecular mechanism of end protection by the 53BP1 pathway. In our opinion, shieldin is likely to represent the ultimate effector of 53BP1, but several key questions must be answered. First and foremost, how does shieldin oppose resection at the molecular level? How does it cooperate with CST? How is it regulated by the cell cycle? Furthermore, orthologues of 53BP1 have been described in many species lacking shieldin (Fig 2B), with the yeast orthologues having been shown to oppose DNA end resection [82,83]. Comparison of the mechanisms behind 53BP1 antagonism of resection in the presence or absence of shieldin will provide valuable insights into the circumstances leading to shieldin evolution.

Other questions of importance relate to potential functions of shieldin outside DSB repair: Is shieldin involved in 53BP1-independent processes such as the regulation of DNA replication timing by RIF1 [84,85] or is it involved in other processes regulated by 53BP1 such as the protection of DNA replication forks [10,11,86]? Finally, the finding that shieldin is critical to mediate the cytotoxicity of PARPi in BRCA1-deficient cells has obvious translational potential. The high incidence of PARPi resistance arising in the clinic, many of which cannot be explained by mutations restoring BRCA1 function, suggests that a variety of factors mediate PARPi lethality in BRCA1-deficient cells [87]. Disruption of any one these factors may subsequently lead to a diminished response to PARPi-based therapy. The studies discussed in this review provide compelling *in vitro* evidence of shieldin being one such factor, but it remains unclear whether loss of shieldin subunits will represent a significant mode of acquired resistance to PARPi in the clinic. Remaining on the topic of PARPi resistance, the ATMIN-DYNLL1 pathway has recently been identified as another mechanism enforcing cytotoxicity of PARPi in *BRCA1*-deficient cells [88,89]. ATMIN is a transcription factor that controls the expression of DYNLL1, a 53BP1-interacting protein, which supports many of its known activities [88,89]). One study proposed that DYNLL1 partially mediates the oligomerization of 53BP1, which is essential for its recruitment to DSBs [89], while another report suggests that DYNLL1 directly interacts with MRN to inhibit its nuclease activity [88]. These findings raise the question of how the mechanisms of shieldin- and DYNLL1-mediated regulation of end resection functionally interact. Does DYNLL1 act upstream or parallel to shieldin, as posited by the oligomerization and MRN inhibitor models, respectively? The answers to these questions will certainly bring us much closer to a long-awaited mechanistic understanding of the regulation of DSB repair in mammalian cells.

Box 1: In need of answers

(i) What is the mechanism of action behind shieldin's inhibition of DNA end resection?
(ii) Is CST necessary for shieldin's function or do the two complexes have distinct contributions to the antagonism of end resection?
(iii) Are there cell-cycle-specific mechanisms that regulate shieldin?
(iv) Is shieldin regulated by TRIP13 through structural remodeling of REV7?
(v) How do 53BP1 orthologues regulate end resection in organisms lacking shieldin?
(vi) Does shieldin have a role in stalled replication fork protection?
(vii) How does shieldin functionally interact with other 53BP1-associated factors such as PTIP, DYNLL1, or TIRR?
(viii) Do shieldin and BRCA1 antagonize each other and if so, what is the mechanism of regulation?

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Conflict of interest

DS has no conflict of interest to declare; DD declares that he is a founder and shareholder of Repare Therapeutics.

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