Cold Spring Harbor Perspectives in Biology
www.cshperspectives.org

Cell Biology of Mitotic Recombination

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Homologous recombination provides high-fidelity DNA repair throughout all domains of life. Live cell fluorescence microscopy offers the opportunity to image individual recombination events in real time providing insight into the in vivo biochemistry of the involved proteins and DNA molecules as well as the cellular organization of the process of homologous recombination. Herein we review the cell biological aspects of mitotic homologous recombination with a focus on *Saccharomyces cerevisiae* and mammalian cells, but will also draw on findings from other experimental systems. Key topics of this review include the stoichiometry and dynamics of recombination complexes in vivo, the choreography of assembly and disassembly of recombination proteins at sites of DNA damage, the mobilization of damaged DNA during homology search, and the functional compartmentalization of the nucleus with respect to capacity of homologous recombination.

Homologous recombination (HR) is defined as the homology-directed exchange of genetic information between two DNA molecules (Fig. 1). Mitotic recombination is often initiated by single-stranded DNA (ssDNA), which can arise by several avenues (Mehta and Haber 2014). They include the processing of DNA double-strand breaks by 5′ to 3′ resection, during replication of damaged DNA, or during excision repair (Symington 2014). The ssDNA is bound by replication protein A (RPA) to control its accessibility to the Rad51 recombinase (Sung 1994, 1997a; Sugiyama et al. 1997; Morrical 2014). The barrier to Rad51-catalyzed recombination imposed by RPA can be overcome by a number of mediators, such as BRCA2 and Rad52, which serve to replace RPA with Rad51 on ssDNA, and the Rad51 paralogs Rad55-Rad57 (RAD51B-RAD51C-XRCC2-XRCC3) and the Psy3-Csm2-Shu1-Shu2 complex (SHU) (RAD51D-XRCC2-SWS1), which stabilize Rad51 filaments on ssDNA (see Table 1 for homologs of yeast and human HR proteins) (Sung 1997b; Sigurdsson et al. 2001; Martin et al. 2006; Bernstein et al. 2011; Liu et al. 2011; Qing et al. 2011; Amunugama et al. 2013; Zelensky et al. 2014). The Rad51 nucleoprotein filament catalyzes the invasion into a homologous duplex to produce a displacement loop (D-loop) (Fig. 1). At this stage, additional antirecombination functions are exerted by Srs2 (FBH1, PARI), which dissociates Rad51 filaments from ssDNA, and Mph1 (FANCM), which disassembles D-loops (see Daley et al. 2014). Upon Rad51-catalyzed strand invasion,

Editors: Stephen Kowalczykowski, Neil Hunter, and Wolf-Dietrich Heyer
Additional Perspectives on DNA Recombination available at www.cshperspectives.org

 doi: 10.1101/cshperspect.a016535
Cite this article as *Cold Spring Harb Perspect Biol* 2015;7:a016535

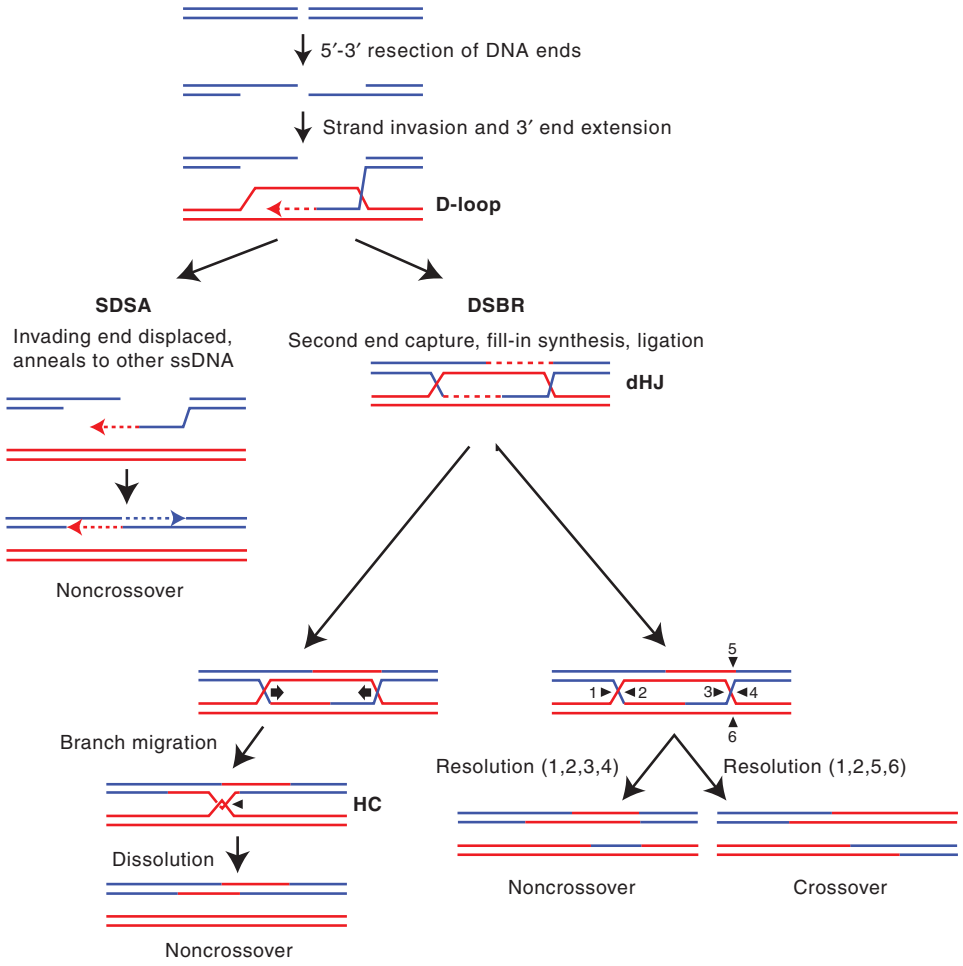

Figure 1. Primary pathways for homology-dependent double-strand break (DSB) repair. Recombinational repair of a DSB is initiated by 5′ to 3′ resection of the DNA end(s). The resulting 3′ single-stranded end(s) invade an intact homologous duplex (in red) to prime DNA synthesis. For DSBs that are repaired by the classical double-strand break repair (DSBR) model, the displaced strand from the donor duplex pairs with the 3′ single-stranded DNA (ssDNA) tail at the other side of the break, which primes a second round of DNA synthesis. After ligation of the newly synthesized DNA to the resected 5′ strands, a double-Holliday junction (dHJ) intermediate is generated. The dHJ can be either dissolved by branch migration (indicated by arrows) into a hemicatenane (HC) leading to noncrossover (NCO) products or resolved by endonucleolytic cleavage (indicated by triangles) to produce NCO (positions 1, 2, 3, and 4) or CO (positions 1, 2, 5, and 6) products. Alternatively to the double-strand break repair (DSBR) pathway, the invading strand is often displaced after limited synthesis and the nascent complementary strand anneals with the 3′ single-stranded tail of the other end of the DSB. After fill-in synthesis and ligation, this pathway generates NCO products and is referred to as synthesis-dependent strand annealing (SDSA).

the ATP-dependent DNA translocase Rad54 enables the invading 3′ end to be extended by DNA polymerases to copy genetic information from the intact duplex (Li and Heyer 2009). Ligation of the products often leads to joint molecules (JMs), such as single- or double-Holliday junctions (s/dHJs) or hemicatenanes (HCs), which must be processed to allow separation of the sister chromatids during mitosis. JMs can be dissolved by the Sgs1-Top3-Rmi1 complex (STR) (BTR, BLM-TOP3α-RMI1-RMI2) (see Bizard and Hickson 2014) or resolved by

Table 1. Evolutionary conservation of homologous recombination proteins between *Saccharomyces cerevisiae* and *Homo sapiens*

Functional class	*S. cerevisiae*	*H. sapiens*
End resection	Mre11-Rad50-Xrs2	MRE11-RAD50-NBS1
	Sae2	CtIP
	Exo1	EXO1
	Dna2-Sgs1-Top3-Rmi1	DNA2-BLM-TOP3α-RMI1-RMI2
Adaptors	Rad9	53BP1, MDC1
	–	BRCA1
Checkpoint signaling	Tel1	ATM
	Mec1-Ddc2	ATR-ATRIP
	Rad53	CHK2
	Rad24-RFC	RAD17-RFC
	Ddc1-Mec3-Rad17	RAD9-HUS1-RAD1
	Dpb11	TOPBP1
Single-stranded DNA binding	Rfa1-Rfa2-Rfa3	RPA1-RPA2-RPA3
Single-strand annealing	Rad52	RAD52
	Rad59	–
Mediators	–	BRCA2-PALB2
	Rad52	–
Strand exchange	Rad51	RAD51
	Rad54	RAD54A, RAD54B
	Rdh54	–
Rad51 paralogs	Rad55-Rad57	RAD51B-RAD51C-RAD51D-XRCC2-XRCC3
	Psy3-Csm2-Shu1-Shu2	RAD51D-XRCC2-SWS1
Antirecombinases	Srs2	FBH1, PARI
	Mph1	FANCM
	–	RTEL
Resolvases and nucleases	Mus81-Mms4	MUS81-EME1
	Slx1-Slx4	SLX1-SLX4
	Yen1	GEN1
	Rad1-Rad10	XPF-ERCC1
Dissolution	Sgs1-Top3-Rmi1	BLM-TOP3α-RMI1-RMI2

structure-selective nucleases, such as Mus81-Mms4 (MUS81-EME1), Slx1-Slx4, and Yen1 (GEN1) (see Wyatt and West 2014). Mitotic cells favor recombination events that lead to noncrossover events likely to avoid potentially detrimental consequences of loss of heterozygosity and translocations.

The vast majority of cell biological studies of mitotic recombination in living cells are performed by tagging of proteins with genetically encoded green fluorescent protein (GFP) or similar molecules (Shaner et al. 2005; Silva et al. 2012). In this context, it is important to keep in mind that an estimated 13% of yeast proteins are functionally compromised by GFP tagging (Huh et al. 2003). By choosing fluorophores with specific photochemical properties, it has been possible to infer biochemical properties, such as diffusion rates, protein–protein interactions, protein turnover, and stoichiometry of protein complexes at the single-cell level. To visualize the location of specific loci within the nucleus, sequence-specific DNA-binding proteins such the Lac and Tet repressors have been used with great success. Specifically, tandem arrays of 100–300 copies of repressor binding sites are inserted within 10–20 kb of the locus of interest in cells expressing the GFP-tagged repressor (Straight et al. 1996; Michaelis et al. 1997). In wild-type budding yeast, such pro-

tein-bound arrays are overcome by the replication fork without a cell-cycle delay or checkpoint activation (Dubarry et al. 2011). However, the arrays are unstable in *rrm3*Δ and other mutants (Dubarry et al. 2011). More pronounced DNA replication blockage by artificial protein-bound DNA tandem arrays has be observed in fission yeast, which is accompanied by increased recombination and formation of DNA anaphase bridges (Sofueva et al. 2011). Likewise, an array of Lac repressor binding sites was reported to induce chromosomal fragility in mouse cells (Jacome and Fernandez-Capetillo 2011). However, these repressor-bound arrays generally appear as a focus with a size smaller than the diffraction limit of light, which is in the range 150–300 nm for wide-field light microscopy.

RECOMBINATION FOCI

In response to DNA damage in eukaryotes, most HR proteins relocalize to nuclear foci of high local concentration at the site of the DNA lesion (Fig. 2) (Lisby et al. 2001, 2003b, 2004; Bekker-Jensen et al. 2006). For example, a single DNA double-strand break (DSB) is sufficient for the formation of a prominent focus containing 600–2100 molecules of Rad52 out of approximately 2300 molecules per haploid cell, yielding a ≥50-fold higher local concentration of Rad52 at the DSB relative to its diffuse nuclear distribution in undamaged cells (Lisby et al. 2003b). Although the minimum number of Rad52 molecules required for mediating a single recombination event is currently unknown (see below), the high local concentration of recombination proteins within these foci may allow constitutively expressed proteins to be active only at the site of DNA damage, and therefore prevent untimely recombination or assembly of recombination complexes at undamaged DNA.

Proteins in recombination foci are highly dynamic, and foci can assemble and disassemble within minutes (Altmannova et al. 2010). The dynamic behavior of recombination foci likely reflects a series of opposing processes, such as Rad52 promoting assembly of Rad51 filaments and Srs2 promoting their disassembly (Boundy-Mills and Livingston 1993; Milne and Weaver 1993; Kaytor et al. 1995; Sung 1997a; New et al. 1998; Shinohara and Ogawa 1998; Burgess et al. 2009). Additional dynamics is likely provided by posttranslational modifications (PTMs), which are continuously applied and erased during repair. Furthermore, studies of Rad51, Rad52, and Rad54 foci in mammalian cells indicate that the residence time of individual molecules may vary between proteins and even subpopulations of proteins within foci (Essers et al. 2002). So far, the dynamics of proteins within individual recombination foci has not been studied in yeast.

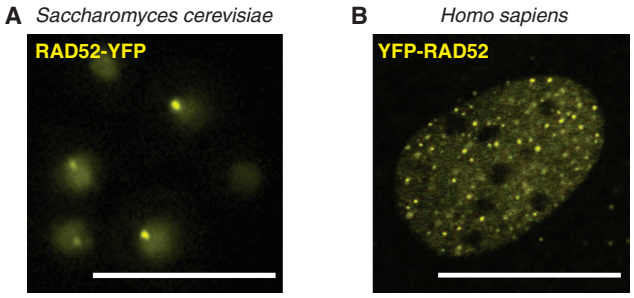

Figure 2. Rad52 foci in yeast and human cells. (*A*) Rad52 foci in *S. cerevisiae*. Cells expressing Rad52-YFP (strain W5094-1C) were exposed to 200 μg/mL of zeocin for 1 h at 25°C. (*B*) RAD52 foci in human cells. The U2OS cell line–expressing YFP-RAD52 were imaged 1 h after exposure to 4 Gy of ionizing radiation, which is equivalent to roughly 50–100 double-strand breaks (DSBs), depending on the phase of the cell cycle. Scale bars, 10 μm. (Image from Bekker-Jensen et al. 2006; reproduced, with permission, from The Rockefeller University Press under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License © 2006.)

CHOREOGRAPHY OF FOCUS FORMATION

The order of assembly of recombination factors at foci during recombinational repair is most extensively studied in the context of DSBs, which will be the topic of this section. Key steps of this assembly process are evolutionarily conserved from yeast to mammals. Hence, the section will focus on yeast and highlight some of the additional complexity and differences reported for other experimental systems (Fig. 3). For an extensive review of DSB-induced foci in mammalian cells, see Thompson (2012). In budding yeast, the Mre11-Rad50-Xrs2 complex (MRX) is the first recombination factor recruited to a DSB, likely by associating directly with DNA ends (Chen et al. 2001; Hopfner et al. 2001; Lisby et al. 2004). The Ku (Yku70-Yku80) complex of the nonhomologous end-joining (NHEJ) pathway can delay recruitment of MRX to DSBs (Lisby et al. 2004). To oppose this effect, it appears that MRX actively promotes displacement of Ku to initiate HR (Wu et al. 2008; Mimitou and Symington 2010). Likely, the competition between Ku and MRX for binding to DSBs is important for determining the choice between NHEJ and HR during DSB repair. Moreover, because MRX is also important for NHEJ (Schiestl et al. 1994; Johzuka and Ogawa 1995; Moore and Haber 1996; Tsukamoto et al. 1996), it is possible that Ku and MRX collaborate to direct the repair of DSBs. In mammalian cells, DNA-PK_{cs} provides an additional barrier to HR (Zhu et al. 2011), and even in G_2 phase, an estimated 80% of ionizing radiation (IR)-induced

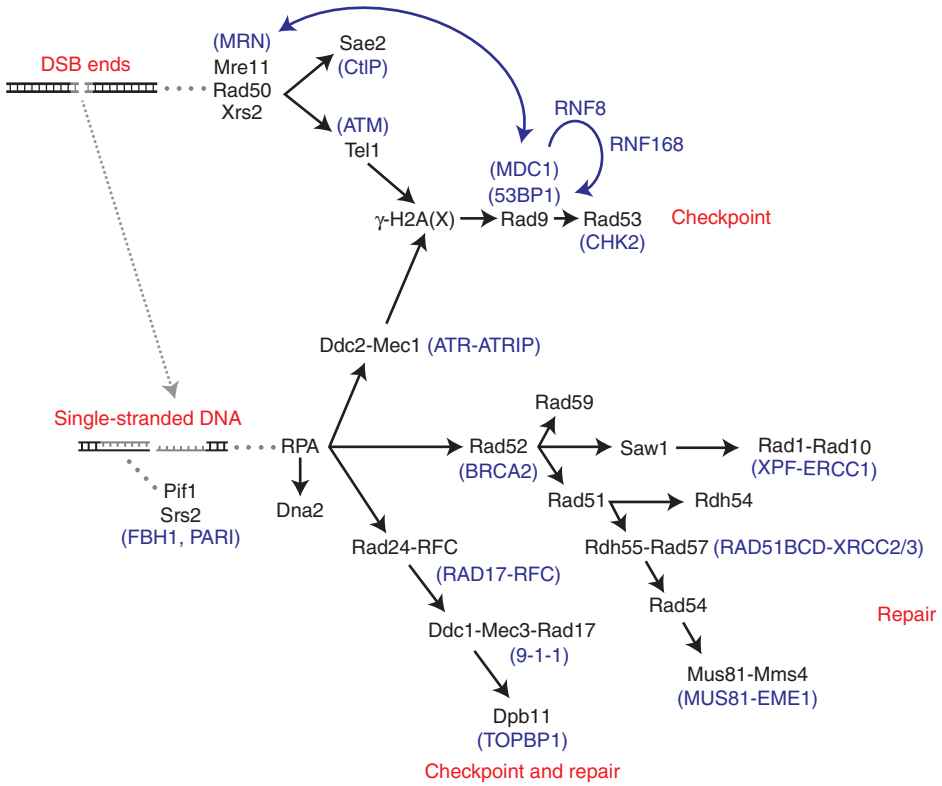

Figure 3. Choreography of homologous recombination (HR) foci in yeast and human cells. Order of recruitment of double-strand break (DSB) repair proteins. Nucleation of proteins at foci start at the *upper left*. Solid black lines represent absolute requirements (see text). Gray dotted lines represent interactions with specific DNA substrates. Arrows indicate protein–protein interactions, modifying functions, and/or feedback loops. Mammalian homologs are indicated in blue.

DSBs are repaired by NHEJ (Beucher et al. 2009; Shibata et al. 2011).

At the cytological level, the two ends of a DSB are tethered by a mechanism that is partially dependent on MRX and Sae2 (Chen et al. 2001; Lisby et al. 2003a; Kaye et al. 2004; Lobachev et al. 2004; Clerici et al. 2005). As a consequence, the two ends of a DSB give rise to a single Mre11 focus rather than two foci. MRX (MRN) and Sae2 (CtIP) physically interact and collaborate in the initial 5′ to 3′ nucleolytic resection of DSB ends (Mimitou and Symington 2009; Yuan and Chen 2009; Ghodke and Muniyappa 2013), which commits the cell to the HR pathway of repair. The Tel1 (ATM) kinase is recruited to DSBs at all phases of the cell cycle via an interaction with Xrs2 (NBS1) (Nakada et al. 2003; Lisby et al. 2004; Falck et al. 2005; You et al. 2005). The Tel1 kinase phosphorylates oligomeric Sae2 to promote its transition to active monomers/dimers, which allows the protein to be recruited to IR-induced foci (Fu et al. 2014). However, Sae2 also forms spontaneous foci, which are independent of MRX, Tel1, and Mec1 (Lisby et al. 2004; Fu et al. 2014). Similarly, in human cells, IR-induced CtIP foci colocalize with MRE11 and NBS1, but these foci of CtIP and MRN appear to form independently (Chen et al. 2008). Further, Tel1 (ATM) phosphorylates histone H2A (H2AX), which is a chromatin mark specific for damaged DNA in most eukaryotes (Rogakou et al. 1998, 1999; Redon et al. 2003; Stiff et al. 2004). Importantly, the modification of chromatin by H2A phosphorylation facilitates binding of the checkpoint adaptor Rad9 to sites of DNA damage likely through a dual interaction of its BRCA1 carboxy-terminal (BRCT) domains with histone H2A phosphorylated at serine 129 (H2A-S129^{P}), and its Tudor domain with histone H3 methylated at lysine 79 (H3-K79^{Me}) leading to subsequent recruitment and activation of Rad53 (Giannattasio et al. 2005; Javaheri et al. 2006; Toh et al. 2006; Grenon et al. 2007; Hammet et al. 2007; Germann et al. 2011). Although the H2A-S129^{P} mark is induced by DNA damage, the H3-K79^{Me} mark is constitutive and thought to be exposed upon damage (Conde et al. 2009). Notably, Rad53 foci are Rad9-dependent, faint, and short-lived, which is consistent with the notion from mammalian cells that CHK2 (Rad53) must redistribute from the site of DNA damage upon phosphorylation to mediate a pan-nuclear checkpoint response (Lukas et al. 2003). Apparently, yeast Rad9 has diverged into MDC1 and 53BP1 in mammalian cells, where MDC1 is recruited to foci via an interaction with γ-H2AX (Stucki et al. 2005) and 53BP1 binds to H3-K79^{Me} (Huyen et al. 2004) and H4-K20^{diMe} (Botuyan et al. 2006). Recruitment of 53BP1 to foci additionally requires its interaction with MDC1 (Eliezer et al. 2009) and with ubiquitylated H2A(X) (Fradet-Turcotte et al. 2013), which is brought about by phosphorylated MDC1 mediating recruitment of the RNF8-RNF168 ubiquitin ligases (Huen et al. 2007; Mailand et al. 2007; Doil et al. 2009; Stewart et al. 2009; Pinato et al. 2011; Mattiroli et al. 2012; Oestergaard et al. 2012; Delgado-Diaz et al. 2014).

The initial short-range resection of DNA ends by MRX-Sae2 is followed by long-range resection by Exo1 and Dna2-STR (DNA2-BTR) (Gravel et al. 2008; Mimitou and Symington 2008; Zhu et al. 2008; Cejka et al. 2010; Niu et al. 2010; Nimonkar et al. 2011; Yan et al. 2011; Symington 2014). Interestingly, the proteins involved in short- and long-range resection have distinct focal appearances giving clues to their function and regulation. Mre11 and Sae2 form prominent nuclear foci at all phases of the cell cycle in response to DSBs (Lisby et al. 2004; Barlow et al. 2008). In contrast, Dna2 shuttles between the nucleus and cytoplasm in a cell-cycle-dependent manner, residing in the cytoplasm during G_1 phase and relocalizing to the nucleus in S/G_2 upon phosphorylation by cyclin-dependent kinase Cdc28 (Kosugi et al. 2009; Chen et al. 2011). Dna2 forms Rad52-colocalizing foci after DSB formation (Zhu et al. 2008). Sgs1 is a low abundance nuclear protein, which forms foci in $S/G_2/M$ (Frei and Gasser 2000; M Wagner, pers. comm.). Exo1 levels are cell-cycle regulated gradually increasing through G_1 and peaking in late S/G_2 phase before it is degraded in anaphase (M Lisby, unpubl.). Resection is accompanied by the dissociation of MRX, Sae2, and Tel1 from DSB-associated foci and binding of RPA to the

3′ single-stranded overhangs (Alani et al. 1992; Lisby et al. 2004; Barlow et al. 2008). In mammalian cells, MRN is retained at DSBs after resection via an interaction with MDC1 (Goldberg et al. 2003; Stewart et al. 2003; Lukas et al. 2004; Lee et al. 2005; Spycher et al. 2008), which binds γ-H2AX, thereby forming a feedback loop for propagation of this histone mark (Stucki et al. 2005). These observations are consistent with the notion that long-range resection is restricted to S/G_2 phases of the cell cycle to allow HR only at this time.

The transition to long-range resection appears to be under tighter control in mammalian cells compared with yeast, which may explain the greater usage of NHEJ for DSB repair in higher eukaryotes. Specifically, 53BP1 and RIF1 inhibit long-range resection (Chapman et al. 2013; Zimmermann et al. 2013). Furthermore, the switch to long-range resection is accompanied by a BRCA1- and POH1-dependent repositioning of 53BP1, RAP80, and K63-linked ubiquitin chains to the periphery of enlarged IR-induced foci (Kakarougkas et al. 2013a), indicating that major reorganization of repair foci takes place to accommodate HR. The intensity of RPA foci can be used to estimate the extent of resection. This approach was used to show at the single-cell level that the rate of DSB end resection increases at the G_1–S transition (Barlow et al. 2008). RPA is necessary for recruiting a number of checkpoint and HR proteins including the Dna2, Mec1-Ddc2 (ATR-ATRIP) (Costanzo et al. 2003; Zou and Elledge 2003; Mailand et al. 2007), Rad24-RFC (RAD17-RFC) (Zou et al. 2002, 2003; Majka and Burgers 2003), and Ddc1-Mec3-Rad17 (9-1-1) complexes (Bermudez et al. 2003; Lisby et al. 2004; Chen et al. 2013). Further, the recruitment of Ddc1-Mec3-Rad17 (9-1-1) to foci is dependent on Rad24-RFC (RAD17-RFC) (Lisby et al. 2004; Medhurst et al. 2008). Notably, Tel1 and Mec1 have many of the same phosphorylation targets, including histone H2A. As a consequence, Tel1-dependent checkpoint signaling is likely replaced by Mec1-dependent signaling upon resection of DSB ends. The multifunctional Dpb11 (TOPBP1) protein is recruited to foci by the 9-1-1 complex (Greer et al. 2003; Germann et al. 2011), reflecting its role in mediating the DNA damage checkpoint through activation of the Mec1 (ATR) kinase (Kumagai et al. 2006; Mordes et al. 2008; Puddu et al. 2008). In contrast, the DNA replication and recombination functions of Dpb11 appear to be independent of focus formation (Germann et al. 2011).

In S and G_2 phase, RPA facilitates the recruitment of Rad52 to DSBs likely via a direct physical interaction (Hays et al. 1995; Lisby et al. 2001, 2004; Plate et al. 2008). The recruitment is independent of DNA replication and requires Cdc28 activity (Barlow and Rothstein 2009). Interestingly, the cell-cycle regulation of Rad52 focus formation can be circumvented at high doses of IR at which Rad52 also forms foci in G_1 phase. However, it is unclear whether these foci are productive for recombination (Lisby et al. 2003a). Rad52 interacts with the Rad51 recombinase and Rad59 to recruit these proteins to foci (Milne and Weaver 1993; Davis and Symington 2003; Lisby et al. 2004). In addition, Rad59 also requires Rad52 for its nuclear localization (Lisby et al. 2004). Despite this requirement, recent data suggest that Rad59 can act independently of Rad52 in genome maintenance (Coic et al. 2008; Pannunzio et al. 2008, 2012).

In mammalian cells, BRCA2 serves as the primary mediator for loading RAD51 onto RPA-coated single-stranded DNA and is required for the formation of IR-induced RAD51 foci (Tarsounas et al. 2003; van Veelen et al. 2005; Badie et al. 2010; Jensen et al. 2010; Liu et al. 2010; Thorslund et al. 2010). RAD51 focus formation is additionally regulated by small ubiquitin-like modifier (SUMO) in both yeast and mammalian cells (Bergink et al. 2013; Shima et al. 2013). It remains to be fully understood how BRCA2 is recruited to foci. However, at least two observations are relevant to this question. First, BRCA2 binds directly to ssDNA (Yang et al. 2002; Jensen et al. 2010; Liu et al. 2010; Thorslund et al. 2010). Second, BRCA2 and PALB2 foci colocalize with BRCA1 foci, and their mutual interdependencies suggest that BRCA2-PALB2 recruitment is initiated by the binding of BRCA1 bound to chromatin in the vicinity of a DSB (Xia et al. 2006). Despite vertebrate RAD52 being dispen-

sable for RAD51 focus formation (van Veelen et al. 2005), the synthetic lethality of *rad52* mutants with mutants of *PALB2*, *BRCA2*, and the RAD51 paralogs *RAD51BCD-XRCC2/3* (Fujimori et al. 2001; Feng et al. 2011; Chun et al. 2013; Lok et al. 2013), indicates a role for RAD52 during HR in higher eukaryotes (Rijkers et al. 1998; Stark et al. 2004).

Together with Rad52, the Rad51 paralogs Rad55-Rad57 promote the formation of Rad51 filaments and themselves require Rad51 for focus formation (Sung 1997b; Lisby et al. 2004). Although the SHU complex—composed of Psy3, Csm2, Shu1, and Shu2—does not relocalize to foci in response to DNA damage in mitotically growing cells, its Csm2 and Psy3 subunits are needed for efficient formation of Rad55 foci (Godin et al. 2013). Similarly, human RAD51B-RAD51C stabilizes the RAD51 nucleoprotein filament (Amunugama et al. 2013), which is consistent with RAD51B-RAD51C providing a mediator function for the assembly of the RAD51-ssDNA nucleoprotein filaments (Sigurdsson et al. 2001). In addition, the Psy3 homolog RAD51D is required for RAD51 focus formation after IR exposure in mammalian cells (Chun et al. 2013). Formation of DNA damage-induced foci of Rad54 requires both Rad55-Rad57 and Rad51, suggesting that Rad54 recruitment to the site of DNA damage requires Rad51 nucleoprotein filament formation (Lisby et al. 2004; van Veelen et al. 2005). Interestingly, the yeast Rad54 homolog, Rdh54, is recruited both to DSBs and to the kinetochore, although the functional significance of this dual localization is unknown. Analysis of Rad51 foci suggests that Rad54 promotes the disassembly of Rad51 from damaged DNA, while Rdh54 disassembles Rad51 from undamaged DNA (Shah et al. 2010). The recruitment of Rdh54 to DSBs is Rad52- and Rad51-dependent, whereas its localization to the kinetochore is independent of the recombination machinery (Lisby et al. 2004). Interestingly, Rad54, which does not localize to the kinetochore in wild-type cells, localizes to the kinetochore in an *rdh54*Δ mutant (Lisby et al. 2004), possibly explaining some of the functional redundancy between these two proteins (Klein 1997; Shinohara et al. 1997). Redundancy is also observed between the RAD54A and RAD54B paralogs in mammalian cells (Tanaka et al. 2000; Wesoly et al. 2006). Furthermore, Rad52 interacts with and recruits Saw1 to foci (Li et al. 2008; Diamante et al. 2014), which in turn facilitates the recruitment of the Rad1-Rad10 endonuclease (Moore et al. 2009). The recruitment of Saw1-Rad1-Rad10 may aid the trimming of recombination structures to promote synthesis-dependent strand annealing (SDSA) (Diamante et al. 2014), a feature that appears to be conserved in mammals (Motycka et al. 2004).

Additional proteins that are recruited to recombination foci include the Pif1 helicase, which forms Rad52-colocalizing foci (Wagner et al. 2006; Pinter et al. 2008), and the Srs2 (FBH1, PARI) helicase and antirecombinase (Krejci et al. 2003; Veaute et al. 2003; Simandlova et al. 2013), which is recruited to two distinct classes of foci (Osman et al. 2005; Burgess et al. 2009; Fugger et al. 2009). During S phase, Srs2 is recruited to sumoylated proliferating cell nuclear antigen (PCNA) speckles, also known as replication foci, and in late S/G_2, Srs2 is recruited to recombination foci marked by Rad52. The recruitment of Srs2 to recombination foci is largely independent of its SUMO-interacting motif (Burgess et al. 2009). The Mus81-Mms4 (MUS81-EME1) structure-selective endonuclease forms foci (Nomura et al. 2007; Matulova et al. 2009), which are largely dependent on Rad54, consistent with Mus81-Mms4 acting downstream from the strand-invasion step of HR and a direct physical interaction between Mus81 and Rad54 (Interthal and Heyer 2000; Matulova et al. 2009). The SUMO-targeted ubiquitin ligase Slx5-Slx8 forms foci that partially overlap with Rad52 and Rad9 foci in response to DNA damage (Cook et al. 2009), and likely reflect the extensive sumoylation of repair proteins at a DSB (Cremona et al. 2012; Psakhye and Jentsch 2012).

RECOMBINATION CENTERS

Cells that experience multiple DSBs have the ability to recruit these lesions to the same repair focus (Fig. 4) (Lisby et al. 2003b; Aten et al.

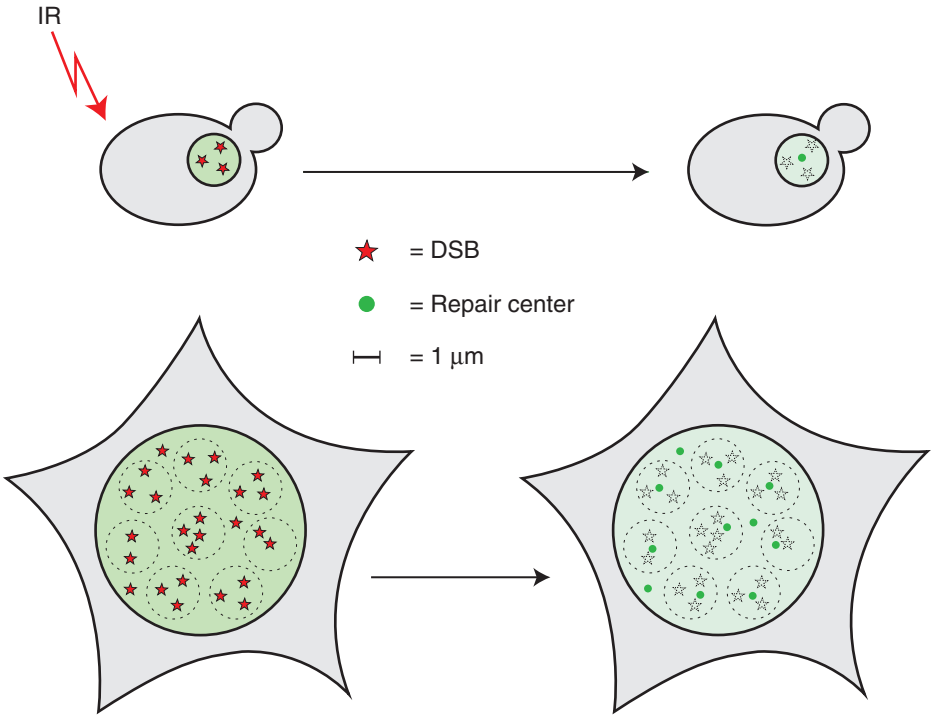

Figure 4. Formation of repair centers in yeast and human cells. The mobility of double-strand breaks (DSBs) induced by ionizing radiation (IR) is confined within the nucleus to a volume having a diameter of 1–2 μm. Because the yeast nucleus is approximately 2 μm in diameter, most DSBs except centromeric and telomeric are essentially free to roam the nucleus and coalesce into repair centers that can be visualize by green fluorescent protein (GFP) tagging of late-acting nuclear recombination proteins (green). The same principle of spatial confinement applies to subregions of mammalian nuclei indicated by dashed circles 2 μm in diameter. Yeast and mammalian cells are illustrated approximately at the same scale.

2004; Neumaier et al. 2012). These recombinational repair centers form in both haploid and diploid yeast cells within 20–30 min of γ irradiation as indicated by the observation that irradiated cells initially show many Mre11 foci within 5 min, which transitions to 1–2 Rad52 foci within 20–30 min (M Lisby, unpubl.). In yeast cells, the aggregation of DSBs is global, whereas it appears to be confined to a volume of 1–2 μm in diameter within the nucleus of mammalian cells, although the mobility of DSBs in mammalian cells seems to vary with cell type and experimental conditions (Aten et al. 2004; Soutoglou et al. 2007; Dimitrova et al. 2008; Jakob et al. 2009; Neumaier et al. 2012; Roukos et al. 2013). Likely, the aggregation of multiple DSBs is the result of PTMs, such as sumoylation and phosphorylation acting as molecular glue to regulate protein–protein interactions at repair foci (Cremona et al. 2012; Psakhye and Jentsch 2012) and/or could be the consequence of the attempt to tether DNA ends. Formation of recombination centers shows a partial dependency on Sae2 and the MRX (MRN) complex for tethering ends (Chen et al. 2001; Lisby et al. 2003a; Kaye et al. 2004; Lobachev et al. 2004; Clerici et al. 2005; Roukos et al. 2013), indicating that DSB end resection is likely a prerequisite for holding ends together.

LIMITATIONS TO FOCIOLOGY

The study of focus formation (fociology) of recombination proteins has proven a useful tool for analyzing the cellular response to DSBs. However, even if a single DSB is sufficient to

trigger focus formation of key recombination proteins (Lisby et al. 2003b), it is likely that some recombination events go undetected by this methodology. For example, recombinational restart of stalled replication forks, some sister chromatid events, and intramolecular recombination may be too fast or require too few molecules of recombination proteins to be detected by current techniques. For example, some proteins (e.g., the SHU complex in mitotic cells) regulate the recruitment of proteins to foci without themselves being detectable at foci (Bernstein et al. 2011). It is worth noting that, in many inducible systems, both sister chromatids are broken, thereby making the DSBs more difficult to repair and allowing more time for proteins to accumulate into foci. Similarly, in mammalian cells, DNA damage-induced protein relocalization after laser microirradiation may be the result of the repair of clusters of DNA breaks in a subnuclear region (Bekker-Jensen et al. 2006).

NUCLEAR COMPARTMENTS

Some genomic sequences are more susceptible to deleterious recombination including repetitive elements, such as the centromeres, telomeres, and Ty elements, and highly transcribed genes, such as the ribosomal gene locus (rDNA) and tRNA genes. Genetic instability at these loci is likely prevented by compartmentalization of the nucleus into domains that suppress recombination and domains that allow or even stimulate recombination. One example is the yeast nucleolus from which late-acting recombination proteins, such as RPA, Rad52, Rad51, Rad59, and Rad55 are largely excluded (Torres-Rosell et al. 2007). DSBs in the rDNA are initially recognized by the MRX complex within the nucleolus and resection is initiated (Torres-Rosell et al. 2007), but they are only bound by Rad52 and downstream factors after relocalization of the break to a position outside the nucleolus. The relocalization of rDNA breaks from the nucleolus to the nucleoplasm requires MRX, the Smc5-Smc6 complex, and SUMO modification of Rad52. Mutations that prevent relocalization lead to Rad52 focus formation inside the nucleolus accompanied by rDNA instability (Torres-Rosell et al. 2007). In human cells, the repair of DSBs in the ribosomal genes has not been studied systematically. However, there are clear indications that most DNA damage response proteins are excluded from the nucleolus (Essers et al. 2002; Bekker-Jensen et al. 2006).

Similarly, budding yeast telomeres are compartmentalized into six to eight clusters (Gotta et al. 1996), which are largely refractory to the DNA damage checkpoint response and recombination (Khadaroo et al. 2009; Ribeyre and Shore 2012). Moreover, telomeres are frequently in the vicinity of the nuclear envelope (Hediger et al. 2002). The anchoring at the nuclear envelope and shielding of telomeres against spontaneous recombination requires the SUN domain protein Mps3 (Antoniacci et al. 2007; Bupp et al. 2007; Oza et al. 2009; Schober et al. 2009; reviewed in Taddei and Gasser 2012). Despite the antirecombinogenic effect of telomere anchoring, their localization at the nuclear periphery is essential for efficient DSB repair in subtelomeric DNA (Therizols et al. 2006). In fact, persistent DSBs, collapsed replication forks, and eroded telomeres associate with the nuclear pore complex (NPC), which stimulates recombinational repair in a Nup84- and Slx8-dependent manner (Nagai et al. 2008; Khadaroo et al. 2009). It was proposed that desumoylation of repair proteins by the SUMO-specific protease Ulp1, which associates with the NPC (Takahashi et al. 2000), could be responsible for the observed stimulation of gene conversion (Nagai et al. 2008). Similarly, in fission yeast, an induced DSB associates with SUN protein Sad1 and KASH protein Kms1 in S/G_2 phases of the cell cycle, connecting the DSB to cytoplasmic microtubules. Via this association, Kms1 and the cytoplasmic microtubule regulator Mto1 promote DSB repair by gene conversion (Swartz et al. 2014). In human cells, the genome is organized into discrete domains by 1300 lamin-associated regions (Guelen et al. 2008), which have been proposed to facilitate some of the genome organization provided by the NPC in yeast (Therizols et al. 2006), which do not have lamins (reviewed in Gonzalo 2014). Mouse telomeres were shown to bind to lamins

and loss of A-type lamins caused a redistribution of telomeres and telomere shortening (Gonzalez-Suarez et al. 2009). A more general role of lamins in the maintenance of genome integrity in higher eukaryotes has been suggested by the increased DNA damage sensitivity and spontaneous γ-H2AX foci associated with mutation of lamins (Liu et al. 2005; Scaffidi and Misteli 2006; di Masi et al. 2008).

Finally, several lines of evidence suggest that DSB repair is differentially regulated in euchromatin and heterochromatin (reviewed in Chiolo et al. 2013) with a preferential repair of heterochromatin DSBs by HR in higher eukaryotes (Shibata et al. 2011; Kakarougkas et al. 2013b). Notably, DNA DSBs in heterochromatin relocalize to euchromatic regions during repair (Jakob et al. 2011), and as a consequence heterochromatic regions often appear to lack radiation-induced γ-H2AX foci (Cowell et al. 2007; Kim et al. 2007; Goodarzi et al. 2008; Vasireddy et al. 2010; Chiolo et al. 2011; Jakob et al. 2011; Lafon-Hughes et al. 2013). The relocalization of heterochromatin DSBs to the periphery of heterochromatic domains is accompanied by a transient ATM-dependent chromatin relaxation (Ziv et al. 2006; Geuting et al. 2013).

DSB DYNAMICS

Early studies suggested that chromosomes move after DNA damage. First, in budding yeast, multiple DSBs accumulate at one or a few repair centers (Lisby et al. 2001), consistent with their movement to a repair center (Lisby et al. 2003b). Also, studies on budding yeast mating type interconversion show DSB-dependent movement of the donor and recipient loci (Houston and Broach 2006; Oza et al. 2009). Evidence from mammalian cell lines after α-particle-induced damage shows that the γ-H2AX foci recruited to the damage coalesce into a few repair centers (Aten et al. 2004). In mouse cells, uncapped telomeres resulting from the ablation of key shelterin component proteins show increased mobility, which is dependent on the checkpoint and repair protein, 53BP1 (Dimitrova et al. 2008). The 53BP1 protein also plays a role during V(D)J recombination stressing the importance of the proper regulation of chromosome mobility (Difilippantonio et al. 2008). Movement of broken chromosomes within the nucleus may facilitate their relocalization from a compartment of low potential for HR to a pro-recombination compartment as detailed in the previous section for rDNA DSBs in budding yeast (Torres-Rosell et al. 2007) and heterochromatic sequences in *Drosophila* (Chiolo et al. 2011). In both cases, relocation depends on the Smc5-Smc6 complex, and in *Drosophila* EXO1 and BLM are required, suggesting that relocalization of fly heterochromatic DSBs requires end resection.

Experiments on broken chromosomes in budding yeast as well as in mammalian cells have shown that DNA damage elicits a change such that the volume of the nucleus explored by the broken chromosome more than doubles (up to as much as 10 times) from that seen in the absence of DSBs (Dion et al. 2012; Krawczyk et al. 2012; Mine-Hattab and Rothstein 2012). In yeast, the chromosomes were marked by multiple tandem arrays, whereas in mammalian cells, the movement was measured by tracking 53BP1, a protein that binds to broken ends to promote DNA repair. The change in mobility of the broken locus is termed "local" or "*cis*." Interestingly, in budding yeast, unbroken chromosomes also increase the volume that they explore after DNA damage, termed "global" or "*trans*" (Mine-Hattab and Rothstein 2012; Seeber et al. 2013). The increase in both global and local mobility in haploid and diploid budding yeast cells is genetically controlled. For example, both depend on the Rad51 recombinase (Dion et al. 2012; Mine-Hattab and Rothstein 2012). In haploid budding yeast, the increase in local mobility depends on Rad54 as well as two checkpoint proteins, Rad9 and Mec1 (Dion et al. 2012; Seeber et al. 2013). In diploid cells, deletion of *SAE2*, which likely causes a delayed appearance of ssDNA, also delays the increase in local chromosome mobility (Mine-Hattab and Rothstein 2012). Unlike the increase in mobility seen after IR or enzymatically induced DSBs, spontaneous Rad52 foci are constrained in haploid cells, possibly reflecting recombination between sister chromatids in the context of DNA

replication (Dion et al. 2013), which may not require increased mobility. In diploid yeast cells, after a DSB, homologous pairing takes approximately 20 min before the repair center disassembles and the loci separate again (Mine-Hattab and Rothstein 2012). Thus, increased chromosomal mobility likely facilitates the homology search, which is otherwise restricted by the proximity of donor and recipient loci (Goldman and Lichten 1996; Agmon et al. 2013; Roukos et al. 2013).

GENOME-WIDE CELL BIOLOGY SCREENS

Whole genome approaches to study proteins involved in the DNA damage response have come to the fore in recent years. Two common kinds of genome-wide cell biology screens have been used to examine the cellular response to DNA damage. In the first, a single tagged protein is introduced into the entire nonessential *S. cerevisiae* gene disruption library to examine the effects of individual deletion mutations on the subcellular behavior of the query protein. In the second, a genome-wide library of GFP-tagged yeast proteins is examined for changes in subcellular localization in response to DNA damage treatment.

In 2007, Alvaro et al. published a screen in *S. cerevisiae* to identify novel factors that impact Rad52 focus formation (Alvaro et al. 2007). The Rad52 protein in *S. cerevisiae* is important for DSB repair and HR (Mortensen et al. 2009). The genes governing Rad52 focus formation and maintenance were not well known when this screen was initiated. To find new genes, the formation of spontaneous subnuclear Rad52-YFP foci was monitored in the mutant background of over 4800 nonessential gene disruptions using epifluorescence microscopy. To avoid the potential problem of additional recessive traits that can accumulate in individual strains of the haploid yeast gene disruption library, hybrid diploid strains that are homozygous for each deletion were made by using systematic hybrid loss of heterozygosity (SHyLOH [Alvaro et al. 2006]). Image analysis was performed manually and all images were uploaded into the JCB DataViewer, which allows anyone to access and examine the primary data from that screen (Thorpe et al. 2011). In this screen, more than 80 gene disruptions resulted in increased spontaneous Rad52 foci, including mutations in many genes involved in DNA metabolism and cell-cycle regulation. The identified genes also included 22 uncharacterized open reading frames, *IRC2–11*, *13–16*, *18–25* (increased recombination centers), providing new leads to genes that control the cellular response to DNA damage.

In mammalian cell studies, two laboratories used a similar approach to identify genes that regulate the DNA damage response. The Cimprich group assayed, using siRNA knockdown, approximately 21,000 genes in HeLa cells for increased γ-H2AX focus formation (Paulsen et al. 2009). Similarly, the Cortez group performed a shRNA screen that targeted almost 2300 genes preselected for protein domains associated with nuclear regulatory activities (Lovejoy et al. 2009). In that study, HeLa cells were treated both with and without aphidicolin to induce replication stress and were assayed for KAP1 phosphorylation, a substrate of the apical ATM kinase. The hits were further validated in U2OS cells by assaying for γ-H2AX foci. In the Cortez study, many of the genes identified were involved in DNA metabolism and repair. In the Cimprich study, in addition to nucleotide excision repair, telomere maintenance, DNA replication, and anaphase-promoting complex-dependent processes, they found that the largest group of genes affecting γ-H2AX focus formation was in mRNA processing or related pathways. Only a few genes overlap between these screens as well as with the yeast results using Rad52 foci as the reporter.

For the second approach, Brown and colleagues measured global changes in the localization and levels of the *S. cerevisiae* library of GFP-tagged proteins in response to DNA damage (Tkach et al. 2012). For their experiments, they tested two drug treatments for the induction of different types of DNA damage. They exposed cells to hydroxyurea (HU), a compound that inhibits DNA replication by limiting the pools of deoxyribonucleotide triphosphates (dNTPs). This resulting dNTP starvation cre-

ates DNA damage. They also used methyl methanesulfonate (MMS) to methylate guanine and adenine residues, which can lead to DSBs (Ma et al. 2008), a very toxic form of DNA damage. They compared the effects of these two drug treatments for each protein with untreated controls by monitoring changes in protein levels of all images using the freely available Cellprofiler software (Kamentsky et al. 2011). They also determined changes in localization by visually comparing each fluorescent strain. Of the more than 4000 tagged proteins screened, they found 356 proteins whose abundance changed significantly after DNA damage, and 254 that changed their localization. Surprisingly, only 35 of these proteins showed changes in both abundance and localization. Furthermore they found that proteins that share a location are also enriched for physical and genetic interactions, which predict common functions, encouraging even more global studies of proteome relocalization in response to other cellular stresses. One of the surprises of their study was that cytoplasmic P-body components, which are important for mRNA turnover in the cytoplasm, become elevated in HU-treated cells. To explore this finding, they next used a genome-wide GFP microscopy assay (similar to the Alvaro et al. study described above) to assess the location of Lsm1, a central P-body component. They found that Asc1, a G-protein β subunit involved in glucose sensing, is specifically required for P-body formation after HU-induced DNA damage, but not after other cellular stresses (Tkach et al. 2012). Importantly they also found that the DNA damage checkpoint kinases Tel1 and Mec1 (ATM and ATR in mammalian cells) inhibit P-body formation. Another surprise is that some proteins (Cmr1, Hos2, Apj1, and Pph21) form DNA damage-induced foci in the nucleus that do not colocalize with repair foci of Rad52. A genome-wide screen of GFP-tagged proteins has also been conducted in *Schizosaccharomyces pombe* (Yu et al. 2013), which identified 51 proteins that were able to form a nuclear focus at a homothallic (HO) switching endonuclease-induced DSB. Eight of these proteins were previously uncharacterized open reading frames (ORFs). All in all, these high-throughput, cell biology-based screens for increased spontaneous DNA damage foci show that repair proteins respond to a variety of genome stress conditions and reveal other pathways not usually associated with the DNA damage response.

PERSPECTIVES

Studies on the cell biology of recombination are still in their infancy. We can look forward to a myriad of technical advances in protein tagging as well as in microscopy (e.g., stochastic optical reconstruction microscopy [STORM], structured illumination microscopy [SIM], HiLo, fluorescence resonance energy transfer [FRET], fluorescence redistribution after photobleaching [FRAP], and single-particle tracking) (Fischer et al. 2011; Lim et al. 2011; Ball et al. 2012; Ishikawa-Ankerhold et al. 2012). These advances when combined with genetic studies will allow a more in-depth look at the architecture of foci as well as provide further insight into the regulation of focus assembly and disassembly. Expansion of genome-wide screens promises to uncover new genes and pathways that impact the cellular response to DNA damage. In the future, more multiple mutant analyses will be undertaken to define the epistasis groups involved. It will be especially important to uncover the genes and regulatory circuitry involved in controlling DNA repair dynamics. Because most live cell–imaging studies only look at a single DNA end, it is important to visualize both ends of a DSB to understand how the homology search is coordinated. Furthermore, DSBs are not the only lesions leading to HR and there is a need to analyze the repair of single-strand nicks and gaps. In addition, methods need to be developed to visualize the underlying changes to the recombining DNA molecules. Another challenge for the future will be to understand which nuclear components are responsible for chromosome territories as well as those that confine diffusion and the mobility of chromosomes. Finally, we can look forward to new insights into the cellular response of recombination processes, especially as they relate to oncogenesis, aging, and other human health issues.

ACKNOWLEDGMENTS

We thank Craig Peterson, Alberto Ciccio, Rune T. Pedersen, Sonia Silva, Eric Bryant, and Michael Smith for insightful comments on the manuscript. We are grateful to the following funding agencies for financial support: Danish Council for Independent Research, the Villum Kann Rasmussen Foundation, and the European Research Council (ERCStG, No. 242905) to M.L., and National Institutes of Health (GM50237 and GM67055) to R.R.

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