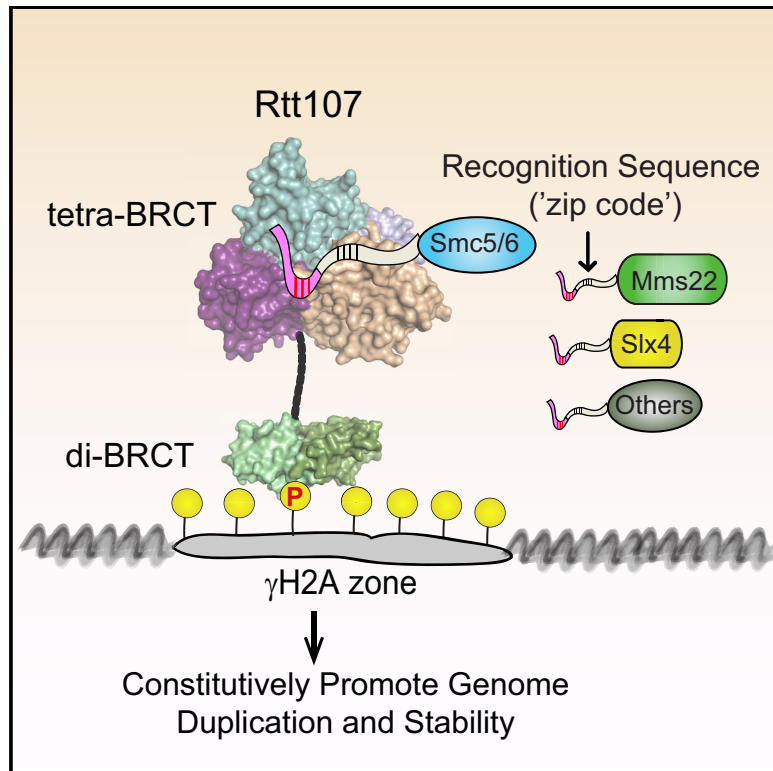


Molecular Basis for Control of Diverse Genome Stability Factors by the Multi-BRCT Scaffold Rtt107

Graphical Abstract



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In Brief

Di-BRCT domains recognize phospho-proteins and contribute to the DNA damage response. Wan et al. show that a distinct architecture formed by four BRCTs of the Rtt107 protein enables recognition of phospho-free ligands and supports constitutive genome protection. This work uncovers structural plasticity and functional diversity of BRCT domains.

Highlights

- Four BRCT units from the Rtt107 protein form a compact higher order assembly
- Rtt107 tetra-BRCT recognizes multiple unmodified ligands through a consensus motif
- Rtt107 binding to genome stability factors provides constitutive genome protection
- Rtt107's tetra- and di-BRCT domains act in concert to recruit proteins to chromatin

Molecular Basis for Control of Diverse Genome Stability Factors by the Multi-BRCT Scaffold Rtt107

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SUMMARY

BRCT domains support myriad protein-protein interactions involved in genome maintenance. Although di-BRCT recognition of phospho-proteins is well known to support the genotoxic response, whether multi-BRCT domains can acquire distinct structures and functions is unclear. Here we present the tetra-BRCT structures from the conserved yeast protein Rtt107 in free and ligand-bound forms. The four BRCT repeats fold into a tetrahedral structure that recognizes unmodified ligands using a bi-partite mechanism, suggesting repeat origami enabling function acquisition. Functional studies show that Rtt107 binding of partner proteins of diverse activities promotes genome replication and stability in both distinct and concerted manners. A unified theme is that tetra- and di-BRCT domains of Rtt107 collaborate to recruit partner proteins to chromatin. Our work thus illustrates how a master regulator uses two types of BRCT domains to recognize distinct genome factors and direct them to chromatin for constitutive genome protection.

INTRODUCTION

Genome maintenance requires a collaborative network of proteins with roles in chromatin replication, repair, and segregation. The architecture of this vast network relies on protein-binding domains that support specific interactions. BRCT (BRCA1 C-terminal) domains, initially identified in the tumor suppressor protein BRCA1, are key constituents of the genome maintenance network across species and support hundreds of protein interactions in human cells (Koonin et al., 1996; Woods et al., 2012). Di-BRCT domains comprising two closely spaced BRCT repeats can recognize phosphorylated proteins, thereby estab-

lishing situation-specific protein interactions critical for the DNA damage response (Manke et al., 2003; Yu et al., 2003). The importance of di-BRCT binding to phospho-peptides is highlighted by its essential role in BRCA1-mediated tumor suppression (Shakya et al., 2011). An open question regarding the underlying principles of BRCT-based networks is whether multi-BRCT domains act as an assembly of di-BRCTs or have unique functions.

To address this question, we investigated the conserved six-BRCT domain-containing Rtt107 protein from budding yeast (Rouse, 2004) (Figure 1A). The most C-terminal two BRCTs (BRCT5 and BRCT6) of Rtt107 and its homologs, including the fission yeast Brc1 and human PTIP, adopt a canonical di-BRCT fold and bind to γ H2A, a phosphorylated form of histone H2A associated with DNA damage and replication sites (Li et al., 2012; Manke et al., 2003; Williams et al., 2010; Yan et al., 2011; Yu et al., 2003). Consistent with this interaction, Rtt107, Brc1, and PTIP promote the DNA damage response (Jowsey et al., 2004; Rouse, 2004; Sheedy et al., 2005). The structures of the four other BRCTs (BRCT1–4) of these proteins and their functional interplay with the corresponding BRCT5 and BRCT6 domains are unclear. As protein interactions underlie the roles of Rtt107 family of factors, addressing these questions is of mechanistic significance.

The Rtt107 region containing BRCT1–4 interacts with the Nse6 subunit of the Smc5/6 complex, the Mms22 subunit of the Cul8 ubiquitin ligase, and the Slx4 protein (Chin et al., 2006; Leung et al., 2011; Roberts et al., 2006; Zappulla et al., 2006). Like Rtt107, these interactors are conserved, and mutations of their human homologs cause DNA fragility, with some leading to genome instability syndromes (Duro et al., 2010; Kim et al., 2011; O'Donnell et al., 2010; van der Crabben et al., 2016). Although members of this Rtt107 interactome promote survival upon exposure to genotoxins via multiple means (Duro et al., 2008; Hang et al., 2015; Ohouo et al., 2013), how Rtt107 interactions affect genome maintenance during normal growth is unclear. Addressing this question will shed important light on Rtt107 interactome functions in continuous genome protection and their human homologs' involvement in diseases.

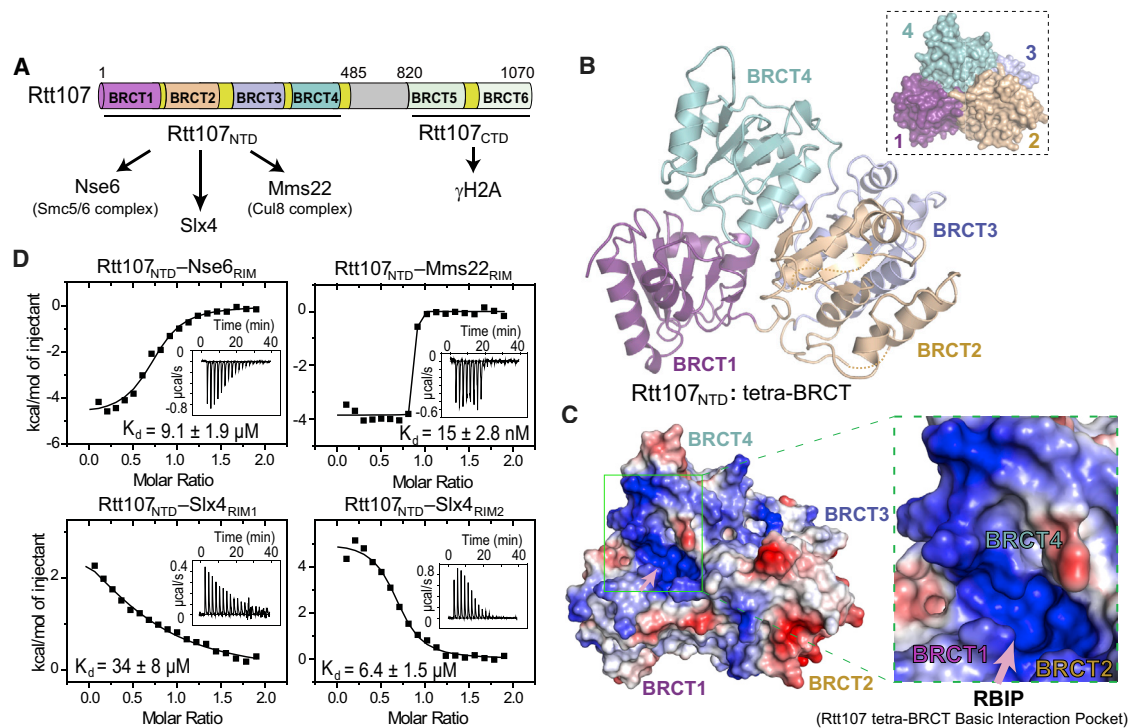


Figure 1. Rtt107_{NTD} Forms a Compact Tetrahedral Structure and Recognizes Phospho-Free Peptide Ligands

(A) Schematic of Rtt107 domains and interacting proteins.
 (B) Ribbon view of the Rtt107_{NTD} structure. Inset shows the surface diagram of the four BRCT repeats, each in a different color.
 (C) Electrostatic surface representation of Rtt107_{NTD}. RBIP is labeled with an arrow in an enlarged view on the right.
 (D) ITC measurement of binding affinities of distinct RIM peptides toward Rtt107_{NTD}.
 See also Figure S1.

Here, we present high-resolution structures of the Rtt107 BRCT1–4 domains with and without bound ligand peptides. These structures reveal a unique BRCT repeat arrangement that enables the recognition of half a dozen unmodified ligands. Cell-based studies uncover a continuous requirement for the Rtt107-client interactions in genome protection and the collaborative nature of its tetra- and di-BRCT domains in targeting client proteins to chromatin. These findings provide structural and molecular basis for how the Rtt107 master regulator recognizes specific genome stability factors and directs them to chromatin for continuous genome protection.

RESULTS

The N-Terminal Four BRCT Repeats of Rtt107 Form a Compact Tetrahedral Architecture

To understand the functional mechanisms of Rtt107 and its interaction network, we first determined the structure of the BRCT1–4 domains of Rtt107 (Rtt107_{NTD}) at a resolution of 2.3 Å (Figures 1B, S1A, and S1B; Table 1). Different from a predicted tandem array of di-BRCTs, Rtt107_{NTD} forms a compact tetrahedral architecture, with individual BRCT units packing into the four apexes of a tetrahedron (Figures 1B and S1C). This intriguing “repeat origami” is enabled by three features. First, in contrast to the parallel repeat arrangement in di-BRCTs (Clapperton et al., 2004;

Shiozaki et al., 2004; Williams et al., 2004), adjacent BRCTs within the tetra-BRCT are rotated relative to each other (Figure S1E). Such an out-of-register repeat configuration makes a higher order folding feasible. Second, inter-BRCT linkers play much more prominent roles in forming the inter-repeat interfaces than linkers in di-BRCTs (Figures S1D and S1F). Such a linker interface provides more freedom in repeat arrangement. Third, BRCT repeats not adjacent in primary sequence also make extensive contacts in Rtt107_{NTD} (Figure S1G). Collectively, these unique features drive the entire Rtt107_{NTD} region to fold into a tight tetrahedral architecture.

It is known that di-BRCT binding to phospho-Ser/Thr depends on a conserved basic site in the first BRCT that consists of a serine or a threonine between β1 and α1 followed by a glycine and a lysine in α2 (Clapperton et al., 2004; Shiozaki et al., 2004; Williams et al., 2004). This site is absent in BRCT1–3 of Rtt107: the equivalent positions of S/T and K are occupied by buried hydrophobic residues, and glycine is replaced by alanine or serine (Figure S1D). BRCT4 of Rtt107 does possess T385 and K426 fitting to a phospho-peptide binding motif, but glycine is replaced by asparagine (Figure S1D). Thus, the four BRCT units in Rtt107_{NTD} do not contain phospho-peptide binding sites as seen in classical di-BRCT domains. Interestingly, a panel of seven positively charged residues in BRCT1 (R107, H108, R110), BRCT2 (R160), and BRCT4 (R392, K425, K426) form a

Table 1. Crystal Data Collection and Refinement Statistics

	Rtt107 _{NTD} (Hg-SAD)	Rtt107 _{NTD} (Native)	Rtt107 _{NTD} -Nse6 _{RIM}	Rtt107 _{NTD} -Mms22 _{RIM}	Rtt107 _{NTD} -Slx4 _{RIM2}
Data Collection					
Space group	P2 ₁	P2 ₁	P2 ₁	P2 ₁	P1
Cell dimensions					
a, b, c (Å)	58.8, 152.9, 76.6	72.4, 98.9, 87.5	71.8, 101.4, 86.6	72.3, 99.7, 167.1	63.5, 78.0, 78.4
α, β, γ (°)	90.0, 106.9, 90.0	90.0, 109.0, 90.0	90.0, 108.6, 90.0	90.0, 94.9, 90.0	90.0, 79.2, 84.0
Resolution (Å)	2.8	2.3	2.4	2.3	1.8
R _{merge}	0.085 (0.406) ^a	0.070 (1.130) ^a	0.086 (0.484) ^a	0.085 (0.982) ^a	0.046 (0.672) ^a
I/σI	11.0 (3.3) ^a	13.3 (1.0) ^a	12.1 (3.1) ^a	21.3 (2.0) ^a	15.0 (2.1) ^a
Completeness (%)	98.3 (99.8) ^a	97.7 (91.0) ^a	99.7 (99.9) ^a	99.1 (97.1) ^a	97.6 (96.1) ^a
Redundancy	3.8 (3.8) ^a	3.7 (3.1) ^a	3.8 (3.8) ^a	3.9 (2.2) ^a	3.9 (4.0) ^a
Refinement					
Resolution (Å)		49.45–2.31	45.71–2.40	49.43–2.30	35.87–1.80
No. of reflections		49,669	45,851	102,648	132,650
R _{work} /R _{free} (%)		21.5/26.6	21.8/27.1	20.4/23.9	15.9/18.5
B factors					
Rtt107 _{NTD}		71.9	47.4	45.0	39.3
RIM		–	79.2	58.3	44.0
Water		64.7	33.9	37.7	44.8
RMSDs					
Bond lengths (Å)		0.008	0.009	0.009	0.006
Bond angles (°)		0.942	1.052	1.168	0.809
Ramachandran Plot					
Favored region		96.0%	95.2%	97.2%	98.5%
Allowed region		100.0%	100.0%	100.0%	100.0%
Outlier region		0.0%	0.0%	0.0%	0.0%

^aHighest resolution shell is shown in parentheses.

deep and large-sized highly basic pocket (Figures 1C, S1D, and S1H). We hypothesized that this prominent feature may contribute to Rtt107 tetra-BRCT interactions through a different mechanism. For simplicity, this deep and basic pocket formed by Rtt107 BRCT1, BRCT2, and BRCT4 is referred to as RBIP (Rtt107 tetra-BRCT basic interaction pocket) hereafter.

Rtt107 Tetra-BRCT Recognizes Multiple Unphosphorylated Peptides

To elucidate how the Rtt107 tetra-BRCT domain associates with genome maintenance factors, including Nse6, Mms22, and Slx4, we first mapped their interaction regions using the yeast two-hybrid (Y2H) assay. Short N-terminal fragments of Nse6 (residues 2–46) and Mms22 (residues 2–38) were found to be necessary and sufficient for Rtt107_{NTD} interactions (Figures S2A and S2B). Intriguingly, Slx4 contains two non-overlapping fragments (residues 407–445 and 535–587) that interacted independently with Rtt107_{NTD} (Figure S2C). We refer to Nse6_{2–46} and Mms22_{2–38} as the Rtt107-interaction motif (RIM) containing regions, or Nse6_{RIM} and Mms22_{RIM}, respectively. Similarly, Slx4_{407–445} and Slx4_{535–587} are referred to as Slx4_{RIM1} and Slx4_{RIM2}, respectively. The four RIM peptides share no sequence homology and are predicted to be unstructured (Figures S2A–S2C). *In vitro*, purified RIM-containing peptides bound to Rtt107_{NTD} with an affinity

ranging from 15 nM in the case of Mms22_{RIM} to 34 μM for Slx4_{RIM1}, as measured by isothermal titration calorimetry (ITC) (Figures 1D and S2D). We conclude that Rtt107 tetra-BRCT binds to Nse6, Mms22, or Slx4 via one or two peptides in the absence of phosphorylation and with a wide range of affinities. To understand how Rtt107 tetra-BRCT recognizes these diverse peptides with different affinities, we proceeded to obtain crystal structures of the different complexes.

The Structure of the Rtt107_{NTD}-Nse6_{RIM} Complex Reveals a Bi-partite Interface

We first determined the Rtt107_{NTD}-Nse6_{RIM} complex structure at a 2.4 Å resolution (Table 1). Rtt107_{NTD} exhibited essentially the same conformation as seen in the ligand-free state (Figure 2A). The electron density map showed that residues 13–19 and 23–41 of Nse6 assumed a well-defined conformation, whereas the linker (residues 20–22) exhibited clear density only for the main chain atoms (Figures 2A, S3A, and S3B). Accordingly, the Rtt107_{NTD}-Nse6_{RIM} interaction entails a bi-partite binding mode with two distinct interfaces, burying 460 and 770 Å² of surface, respectively.

The N-terminal region of Nse6_{RIM} (residues 13–19; Nse6_{RIM-N}) packs along one side of BRCT4 of Rtt107 through main chain hydrogen-bonding interactions and points into RBIP that we

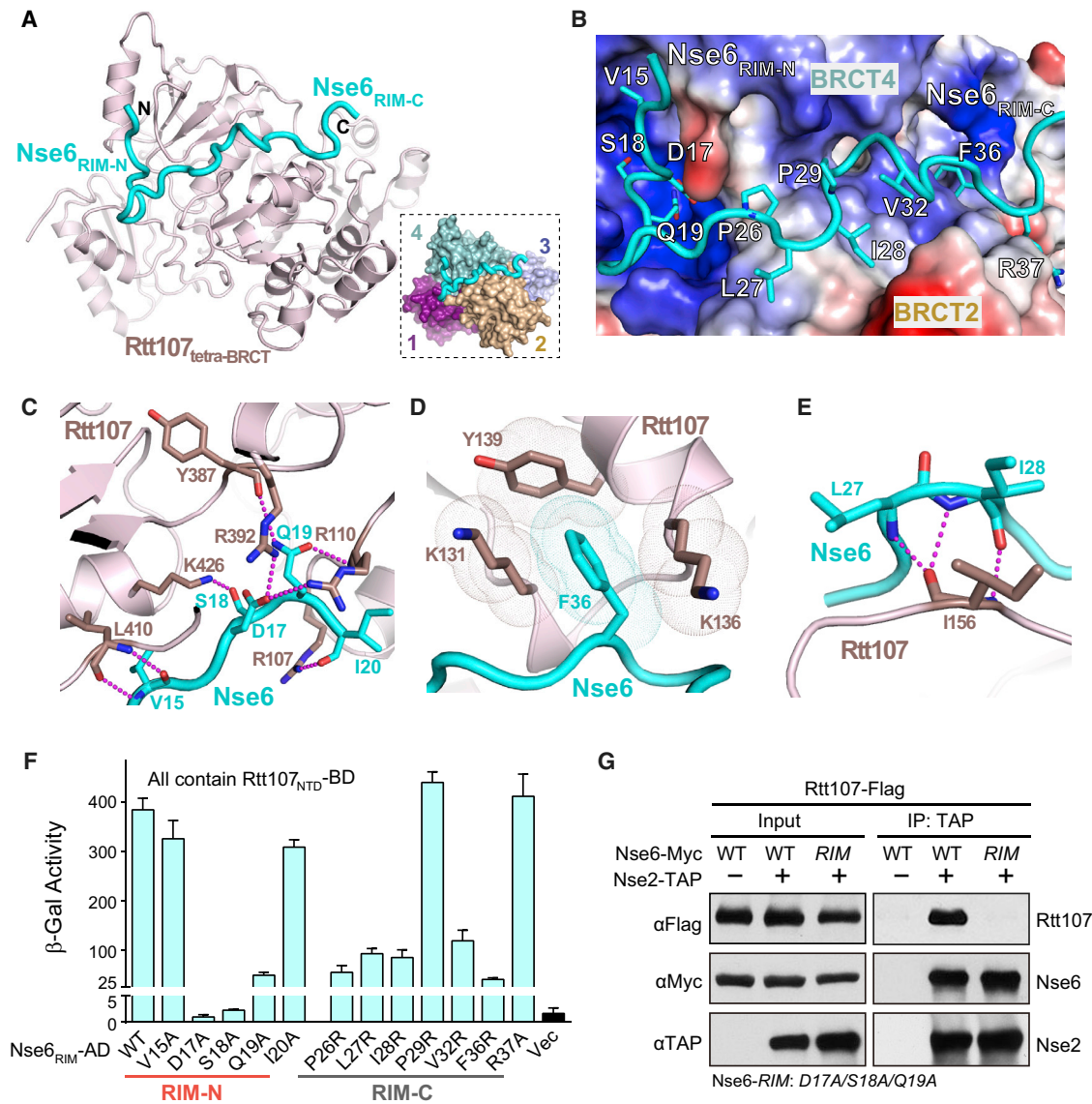


Figure 2. The Structure of the Rtt107_{NTD}-Nse6_{RIM} Complex Reveals a Bi-partite Interface

(A) Overall structure of Rtt107_{NTD} (pink) bound to Nse6_{RIM} (cyan). A surface diagram is shown in the inset.

(B) The Nse6_{RIM} peptide forms two interfaces with the Rtt107 tetra-BRCT. Electrostatic surface of Rtt107_{NTD} and key Nse6_{RIM} contacts are shown.

(C–E) Close-up views of the Nse6_{RIM} and Rtt107_{NTD} interface. (C) The Nse6_{RIM-N} D_{SQ}_{17–19} motif forms multiple electrostatic interactions with Rtt107 BRCT1 and BRCT4 residues. (D) The side chain of F36 in Nse6_{RIM-C} forms hydrophobic interactions with three Rtt107 residues. (E) Main chain hydrogen-bonding interactions between Nse6_{RIM-C} and Rtt107_{NTD}.

(F) The effects of mutating Nse6 residues on the Rtt107_{NTD} Y2H interaction. Y2H reporter assay data measuring β-galactosidase activities are represented for Rtt107_{NTD} paired with wild-type and mutated peptides. For each pair, the data were quantified from three transformants with average and SD shown.

(G) Nse6-*RIM* abolishes the interaction with Rtt107, but not with Nse2. Immunoprecipitation of TAP-tagged Nse2 pulled down both wild-type Nse6 and Nse6^{*RIM*} mutant proteins, but only pulled down Rtt107 if Nse6 was wild-type.

See also Figures S2–S4.

hypothesized as a potential protein interaction site (Figure 2B). At the bottom of this pocket, residues D_{SQ}_{17–19} of Nse6 mediate an extensive electrostatic network with Rtt107_{NTD}: the side chain of D17 forms two salt bridges with Rtt107^{R110}_{NTD} and Rtt107^{R392}, while the S18 side chain forms a hydrogen bond with Rtt107^{K426} and the Q19 side chain coordinates two hydrogen bonds with Rtt107^{R110} and Rtt107^{Y387} (Figure 2C).

In contrast to Nse6_{RIM-N}, the C-terminal region of Nse6_{RIM} (residues 23–41; Nse6_{RIM-C}) adopts an extended conformation meandering along a shallow hydrophobic groove between BRCT2 and BRCT4 of Rtt107 (Figure 2B). Near the C-terminal end of Nse6_{RIM-C}, the F36 side chain is embedded in a hydrophobic pocket formed by Rtt107^{K131}, ^{K136}, ^{Y139} (Figure 2D). Additionally, multiple main chain hydrogen-bonding interactions

strengthen the Nse6_{RIM-C}-Rtt107_{NTD} interface (Figure 2E). Together, these elaborate interactions extend the contact interface and contribute to the specific recognition of the Nse6_{RIM} peptide by Rtt107_{NTD}.

Consistent with our structural data, Y2H assays showed that individual alanine substitutions of Nse6_{RIM-N} residues D17, S18, and Q19, which constitute an electrostatic interface with Rtt107_{NTD}, abolished or greatly reduced Y2H interactions with Rtt107 (Figure 2F). In addition, mutations of Nse6_{RIM-C} residues that contribute to the hydrophobic contacts with Rtt107_{NTD} reduced the interaction, with F36R showing the strongest defect (Figure 2F). These data support that Rtt107_{NTD} associates with Nse6_{RIM} using a bi-partite recognition mechanism, with electrostatic contact of Nse6_{RIM-N} being more critical than the hydrophobic contact of Nse6_{RIM-C}.

We next examined the consequences of alanine substitution of the Nse6 DSQ₁₇₋₁₉ motif (referred to as Nse6^{RIM}) in the context of the endogenously expressed full-length protein. Nse6^{RIM} maintained its association with Nse2, another subunit of the Smc5/6 complex, but failed to interact with Rtt107 in co-immunoprecipitation experiments (Figure 2G). Hence, the observed electrostatic interface between Nse6 and Rtt107 is necessary for their interaction *in vitro* and *in vivo*.

The Rtt107_{NTD}-Mms22_{RIM} Interaction Also Uses a Bi-partite Mode but Has Distinct Features

We moved on to examine Mms22_{RIM}. Guided by our structural understanding of Nse6_{RIM}, we found that Mms22_{RIM} shares an uneven distribution of amino acids as seen in Nse6_{RIM}, including a highly acidic N-terminal half and a hydrophobic C-terminal half (Figures S4A and S4B). This raised the possibility that Mms22_{RIM} might use a bi-partite mode to interact with Rtt107_{NTD} similar to Nse6_{RIM}. To test this premise, we solved the Rtt107_{NTD}-Mms22_{RIM} complex structure (Figure 3A; Table 1). Remarkably, the C-terminal hydrophobic region of Mms22_{RIM} (Mms22_{RIM-C}; residues 22–37) binds to the same hydrophobic groove between BRCT2 and BRCT4 of Rtt107_{NTD} as Nse6_{RIM-C} (Figures 3B; Figures S3C and S3D).

Despite the overall similarity, the local landscapes of the Rtt107_{NTD} hydrophobic groove in the two complexes are substantially different (Figure 3C). In the Rtt107_{NTD}-Nse6_{RIM} complex, the side chains of Rtt107^{K136, K373} protrude into the central space as a crossbeam in the groove, forming part of the hydrophobic pocket for F36 of Nse6 and forcing the Nse6_{RIM} C terminus toward BRCT2 of Rtt107 (Figure 3C, top). In contrast, side chains of Rtt107^{K136, K373} in the Rtt107_{NTD}-Mms22_{RIM} interface rotate away from the groove, resulting in an enlarged hydrophobic cavity to accommodate side chains of L34 and W35 of Mms22 (Figure 3C, bottom). Notably, the side chain of Y33 of Mms22 occupies the same hydrophobic pocket as F36 of Nse6, although in an opposite orientation and forming more extensive interactions with Rtt107 (Figures 3C and 3D). As a consequence of this local rearrangement, the interface in the Rtt107_{NTD}-Mms22_{RIM-C} complex is extended to 920 Å² from 770 Å² in the Rtt107_{NTD}-Nse6_{RIM-C} complex, explaining the stronger binding affinity of Mms22_{RIM} toward Rtt107_{NTD} (Figure 1D).

Although the acidic N-terminal region of Mms22_{RIM} (Mms22_{RIM-N}) is not visible in the electron density map, its prox-

imity to RBIP and their oppositely charged natures suggest that these two regions are closely associated. We used mutagenesis analyses to test this premise. Y2H data showed that like Mms22_{RIM-C}, Mms22_{RIM-N} was necessary for Rtt107 binding (Figure S4B). Moreover, point mutations of DSE₁₃₋₁₅, which resembles the Nse6_{RIM-N} DSQ₁₇₋₁₉ motif that contacts RBIP (Figures S4A and S4B), reduced the Y2H Rtt107_{NTD} interaction, with the D13 mutations having the strongest effects (Figure 3E). A reduction in interaction was also observed upon mutating the Mms22_{RIM-C} hydrophobic contacts with Rtt107_{NTD} (Figure 3E). Only when D13A was combined with Y33A, L34A, or W35A, the Mms22_{RIM} and Rtt107_{NTD} interaction was abolished (Figure 3E). This result suggests that electrostatic and hydrophobic contacts are both important for the Rtt107-Mms22 interaction, differentiating this interaction from that of Rtt107 and Nse6. Testing one of the combined Mms22 mutations, D13A/Y33A (mms22^{RIM}), in the context of the endogenously expressed full-length protein showed that its association with Rtt107, but not with the Mms1 subunit of the Cul8 ubiquitin E3 complex, was disrupted (Figure 3F). Thus, we conclude that Mms22 also uses a bi-partite mode to interact with Rtt107_{NTD}, analogously to Nse6; however, the relative contributions of the acidic versus hydrophobic sequences of their RIMs are different.

Shared and Unique Features of Rtt107 Interaction with Slx4_{RIM1} and Slx4_{RIM2}

Our findings that the Rtt107 tetra-BRCT domain recognizes Nse6 and Mms22 RIMs using a similar bi-partite mode raised the possibility that this may be a general principle for ligand recognition by this tetra-BRCT. To test this hypothesis, we examined the Slx4 RIMs. The Slx4_{RIM1} sequence has a bi-partite structure similar to Nse6_{RIM} and Mms22_{RIM} (Figure S4C). Slx4_{RIM1-N} contains a DTT₄₂₂₋₄₂₄ motif mimicking the DSE/Q motif in Nse6_{RIM-N} and Mms22_{RIM-N}, while Slx4_{RIM1-C} contains a stretch of hydrophobic residues similar to Nse6_{RIM-C} and Mms22_{RIM-C} (Figures S4A–S4C). Importantly, as seen for Nse6_{RIM}, mutation of the DTT₄₂₂₋₄₂₄ motif abolished the Y2H interaction with Rtt107, while mutation of hydrophobic residues partially reduced the interaction (Figure 4A). These data suggest that Slx4_{RIM1} also uses a bi-partite mode for associating with Rtt107_{NTD}, but it resembles Nse6_{RIM} more than Mms22_{RIM} in that the D-S/T-Q/E/T motif makes a major contribution to the interaction.

Slx4_{RIM2} does not have a bi-partite sequence as seen in the other three RIMs but rather contains a stretch of acidic residues in its C-terminal region (Figure S4C). To understand how Slx4_{RIM2} is recognized by Rtt107_{NTD}, we determined Rtt107_{NTD}-Slx4_{RIM2} complex structure (Figure 4B; Table 1). Unlike the extended conformations of the other RIMs, Slx4_{RIM2} forms a compact fold with a three-stranded β sheet covered by a short α-helix (Figures 4B; Figures S3E and S3F). Remarkably, this structure sits right on top of the hydrophobic groove between Rtt107's BRCT2 and BRCT4 repeats, which also binds Nse6_{RIM-C} and Mms22_{RIM-C} (Figure 4C). Several Slx4_{RIM2} residues, including I538, V540, L550, and L554, show hydrophobic interactions with Rtt107 in this groove (Figure 4C). Consistently, mutating these residues weakened the Y2H interaction with Rtt107_{NTD} (Figure 4D). Compared with the interfaces in the Rtt107_{NTD}-Nse6_{RIM-C} and Rtt107_{NTD}-Mms22_{RIM-C} complexes, Slx4_{RIM2} partially occupies

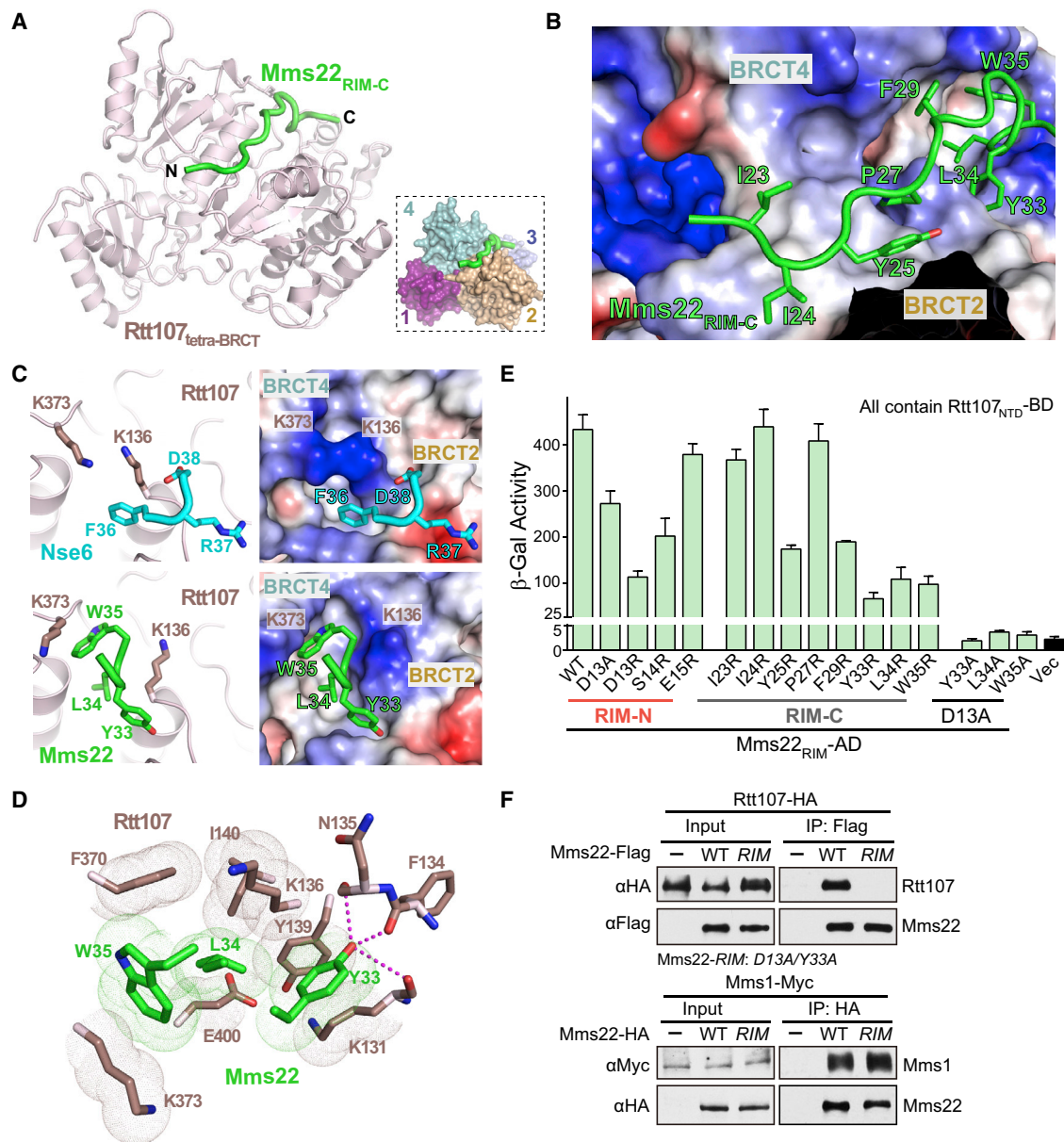


Figure 3. The Rtt107_{NTD}-Mms22_{RIM} Interaction Entails a Bi-partite Mode but Has Distinct Features

(A) Overall structure of the Rtt107_{NTD} (pink) bound to Mms22_{RIM} (green).

(B) Mms22_{RIM-C} binds to a hydrophobic groove formed by Rtt107_{NTD} BRCT2 and BRCT4. Mms22_{RIM-C} peptide (green, stick) and the Rtt107_{NTD} groove (electrostatic surface) are shown.

(C) Comparison of hydrophobic interfaces between the Rtt107_{NTD}-Nse6_{RIM} complex and the Rtt107_{NTD}-Mms22_{RIM} complex. Rtt107_{NTD} is shown as ribbon (left) and electrostatic surface representation (right).

(D) Close-up views of the interface between Rtt107_{NTD} and Mms22_{RIM-C}. Hydrophobic interactions and hydrogen bonds are indicated by dots and dashed lines, respectively.

(E) Summary of the effects of Mms22 mutations on the Rtt107_{NTD} Y2H interaction. Y2H reporter assay data are presented as in Figure 2F.

(F) Mms22-RIM abolishes the interaction with Rtt107 but not with Mms1 *in vivo*.

See also Figures S2-S4.

the hydrophobic groove of Rtt107_{NTD}, with only a 540 Å² interface area between Rtt107_{NTD} and Slx4_{RIM2}.

Intriguingly, the acidic stretch in the Slx4_{RIM2} C-terminal region is within an extended loop between strands β 2 and β 3 and fits right

into RBIP (Figure 4C). This interface entails an extensive hydrogen-bonding network, with S567, E569, E571, and T572 of Slx4 forming multiple contacts with Rtt107^{K107, Q126, R160} (Figure 4E). Furthermore, S567 of Slx4 coordinates this network by

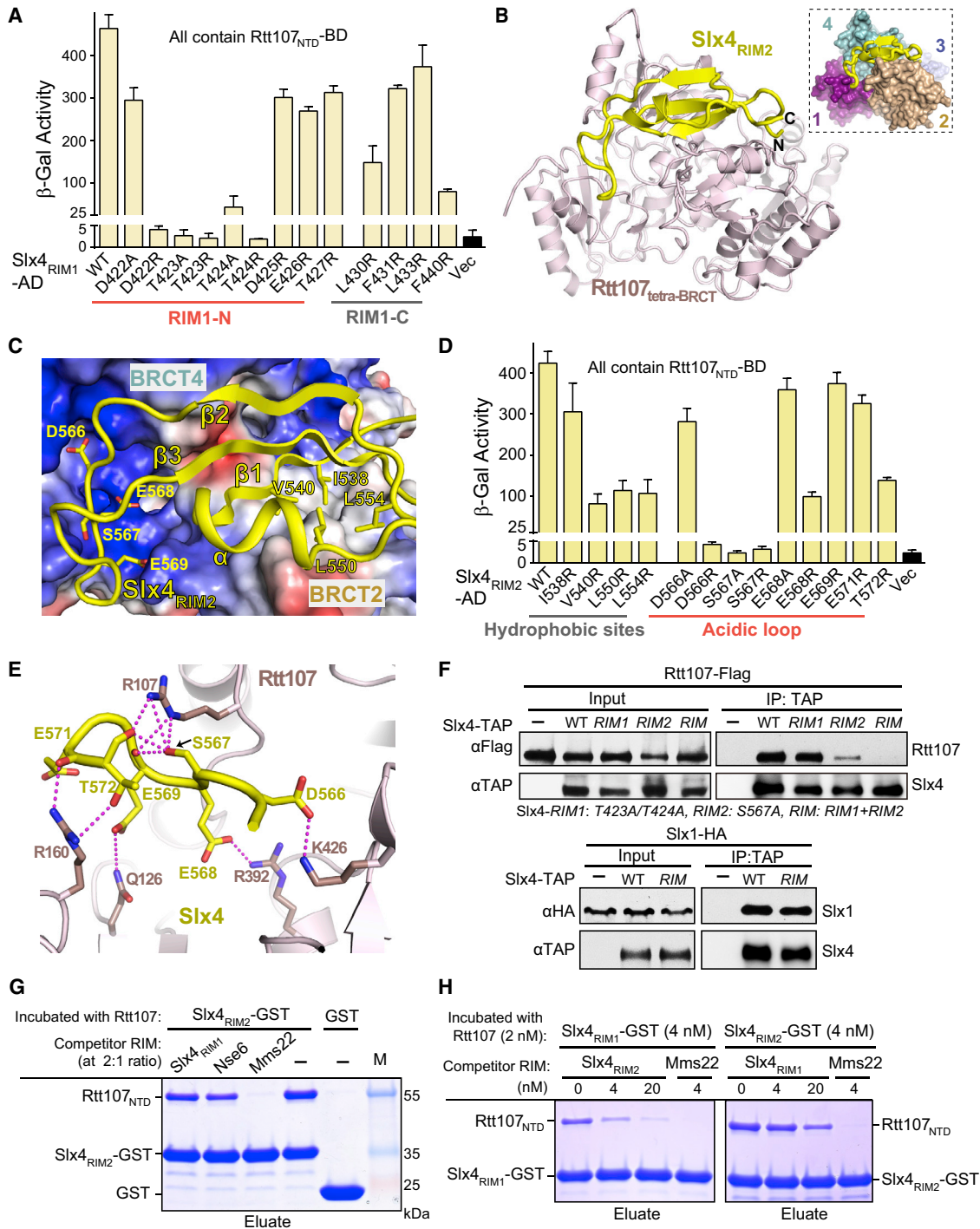


Figure 4. Shared and Unique Features of Rtt107 Interactions with Two Slx4 Regions

(A) Effects of Slx4_{RIM1} mutations on the Rtt107_{NTD} Y2H interaction. Y2H reporter assay data are presented as in Figure 2F.
 (B) Overall structure of Rtt107_{NTD} (pink) bound to Slx4_{RIM2} (yellow).
 (C) The interface between Slx4_{RIM2} and Rtt107_{NTD}. Rtt107_{NTD} is shown in electrostatic surface representation and Slx4_{RIM2} in ribbon view.
 (D) Effects of Slx4_{RIM2} mutations on the Rtt107_{NTD} Y2H interaction. Y2H reporter assay data are presented as in Figure 2F.
 (E) Close-up view of the electrostatic interaction between Slx4_{RIM2} and Rtt107_{NTD}.
 (F) Mutating residues of the two Slx4 RIM regions affects the association with Rtt107 but not with Slx1 as examined by co-immunoprecipitation.

(legend continued on next page)

forming intramolecular interactions with E569 and T572 (Figure 4E). Adjacent to this, D566 and E568 of Slx4 form electrostatic interactions with Rtt107^{K426} and Rtt107^{R392}, respectively (Figure 4E). Such an extensive electrostatic network likely compensates for the relatively smaller hydrophobic interface between Slx4_{RIM2} and Rtt107 to support a stable interaction. Notably, the DSE₅₆₆₋₅₆₈ motif within the Slx4_{RIM2} acidic loop fits the D-S/T-Q/E/T consensus in other RIM-N peptides examined. Consistent with a key structural role, the S567A mutation of Slx4_{RIM2} abolished Y2H interaction with Rtt107_{NTD}, while D566R and E568R reduced this interaction (Figure 4D). We thus conclude that Slx4_{RIM2} adopts a different conformation from Nse6_{RIM} and Mms22_{RIM}, but still associates with Rtt107_{NTD} via the same bi-partite mechanism.

To generate a *slx4* mutant that abolishes Rtt107 association, we tested T423A/T424A in Slx4_{RIM1} and S567A in Slx4_{RIM2}, which individually exhibited strong Rtt107_{NTD} interaction defects (Figures 4A and 4D). In cells, Slx4-S567A showed more severe defects in Rtt107 association than Slx4-T423A/T424A, and their combined mutation (referred to as *slx4*^{RIM}) completely abolished the Rtt107-Slx4 interaction (Figure 4F). This effect is specific as *slx4*^{RIM} did not change the interaction with another known interactor, Slx1 (Figure 4F).

Mutually Exclusive RIM Binding of Rtt107_{NTD}

Our conclusion that the Rtt107 tetra-BRCT uses similar mechanisms to recognize different RIM sequences suggests mutually exclusive interactions such that RIM peptides compete with each other for Rtt107 binding. Our *in vitro* competition assay results indeed supported this prediction. We challenged the GST-Slx4_{RIM2}-Rtt107_{NTD} complex with a competitor RIM peptide at 2-fold molar excess and examined the remaining bead-bound complex (Figure S5A). Compared with a control reaction to which no competitor was added, bead-bound Rtt107_{NTD} levels were greatly reduced by the addition of Mms22_{RIM} and moderately by Nse6_{RIM} (Figures 4G and S5B). The different effects are in line with the stronger affinity of Mms22_{RIM} for Rtt107_{NTD} compared with Nse6_{RIM} and Slx4_{RIM2} (Figure 1D). Less Rtt107_{NTD} was released from GST-Slx4_{RIM2} upon addition of Slx4_{RIM1}, compared with Mms22_{RIM} and Nse6_{RIM}; however, this was improved when Slx4_{RIM1} was added 5-fold in excess (Figures 4H and S5C). In reciprocal experiments, Slx4_{RIM2} effectively released Rtt107_{NTD} bound to GST-Slx4_{RIM1} (Figures 4H and S5C). These results agree with our data that Slx4_{RIM2} binds more strongly to Rtt107_{NTD} than Slx4_{RIM1} and they occupy the same sites on Rtt107_{NTD}. Data above provided biochemical validation of the conclusion that Rtt107_{NTD} recognizes different RIM sequences by similar mechanisms.

Additional RIM-Containing Proteins Expand the Scope of the Rtt107 Tetra-BRCT Interactome

Identification of RIM sequences above enabled us to examine the scope of RIM-mediated Rtt107 interactions in the proteome. We used the most conserved feature of the RIMs, namely, the

D-S/T-Q/E/T motif present in an acidic loop, to examine reported Rtt107 Y2H interactors (Chin et al., 2006; Princz et al., 2017). We identified DSE₁₈₇₋₁₈₉ in the centromeric protein Scm3, DSN₃₈₅₋₃₈₇ in the homologous recombination protein Rad55, and EST₄₈₂₋₄₈₄ in the Cdc7 kinase, all in unstructured acidic regions (Figure S5D). Significantly, a peptide containing this motif from each of these proteins bound to Rtt107 *in vitro* (Figure S5E). These peptides also interacted with Rtt107_{NTD} in Y2H analysis in a manner dependent on the basic residues of RBIP, as in the case for Nse6_{RIM} and Slx4_{RIM1} (Figure S5F). In addition, these Y2H interactions required the D-S/T-Q/E/T motif of Scm3, Rad55 and Cdc7 (Figure S5G). These data suggest that these three proteins interact with Rtt107 using a similar mechanism as Nse6, Slx4, and Mms22, highlighting the prominent role of the D/E-S/T-Q/E/T/N motif for mediating Rtt107 tetra-BRCT association with partner proteins.

RIM Mutants Are Separation-of-Function Alleles that Lead to Genomic Instability during Growth

After establishing the structural basis of Rtt107 tetra-BRCT interactions with client proteins, we addressed the functional significance of these interactions. Rtt107 associates separately with Nse6, Mms22, and Slx4 during growth and after DNA damage (Hang et al., 2015). However, it has been difficult to pinpoint the roles of these interactions in genome protection, particularly during growth as their null or hypomorphic alleles exhibit pleiotropic defects (Aragón, 2018; Cussiol et al., 2017; Duro et al., 2008; Wan et al., 2016). Because RIM mutants (*nse6*^{RIM}, *mms22*^{RIM}, and *slx4*^{RIM}) described above disrupted the associations of their corresponding proteins with Rtt107 but not with other interactors, they likely specifically abolish the functions mediated by interactions with Rtt107. This notion was validated genetically, as RIM mutants did not worsen the growth or genotoxin sensitivity of *rtt107Δ* cells, unlike *mms22Δ*, *slx4Δ*, or *smc6-P4* hypomorphic allele (*Smc5/6* is essential) (Figure 5A). On the basis of our biochemical and genetic data, we conclude that RIM mutants are separation-of-function alleles uniquely able to reveal the consequences of disrupting the Rtt107 tetra-BRCT interactions with the corresponding proteins. We note that combining the RIM mutations of Slx4, Mms22, and Nse6 conferred less genotoxin sensitivity than *rtt107Δ* (Figure S6A). This finding is consistent with our data that the tetra-BRCT of Rtt107 also interacts with other proteins that contribute to genome protection as described above.

We used gross chromosomal rearrangement (GCR) assay (Putnam et al., 2009) to study the effects of RIM mutants on overall genome stability during growth. We found that *nse6*^{RIM}, *mms22*^{RIM}, and *slx4*^{RIM} mutants exhibited 3- to 34-fold increased rates over wild-type levels, while *rtt107Δ* exhibited an even higher rate (Figure 5B). Thus, Rtt107 interactions with Nse6, Mms22, and Slx4 suppress genome instability during continuous growth to different degrees.

(G and H) *In vitro* competition assays among RIM peptides. In (G), a pre-formed complex of Rtt107_{NTD} and Slx4_{RIM2}-GST was challenged by the Slx4_{RIM1}, Nse6_{RIM} or Mms22_{RIM} peptide. In (H), a pre-formed complex of Rtt107_{NTD} and Slx4_{RIM1}-GST or Rtt107_{NTD} and Slx4_{RIM1}-GST was challenged by the competitor peptides as indicated. Experimental procedures and input protein levels are included in Figures S5A–S5C. See also Figures S2–S5.

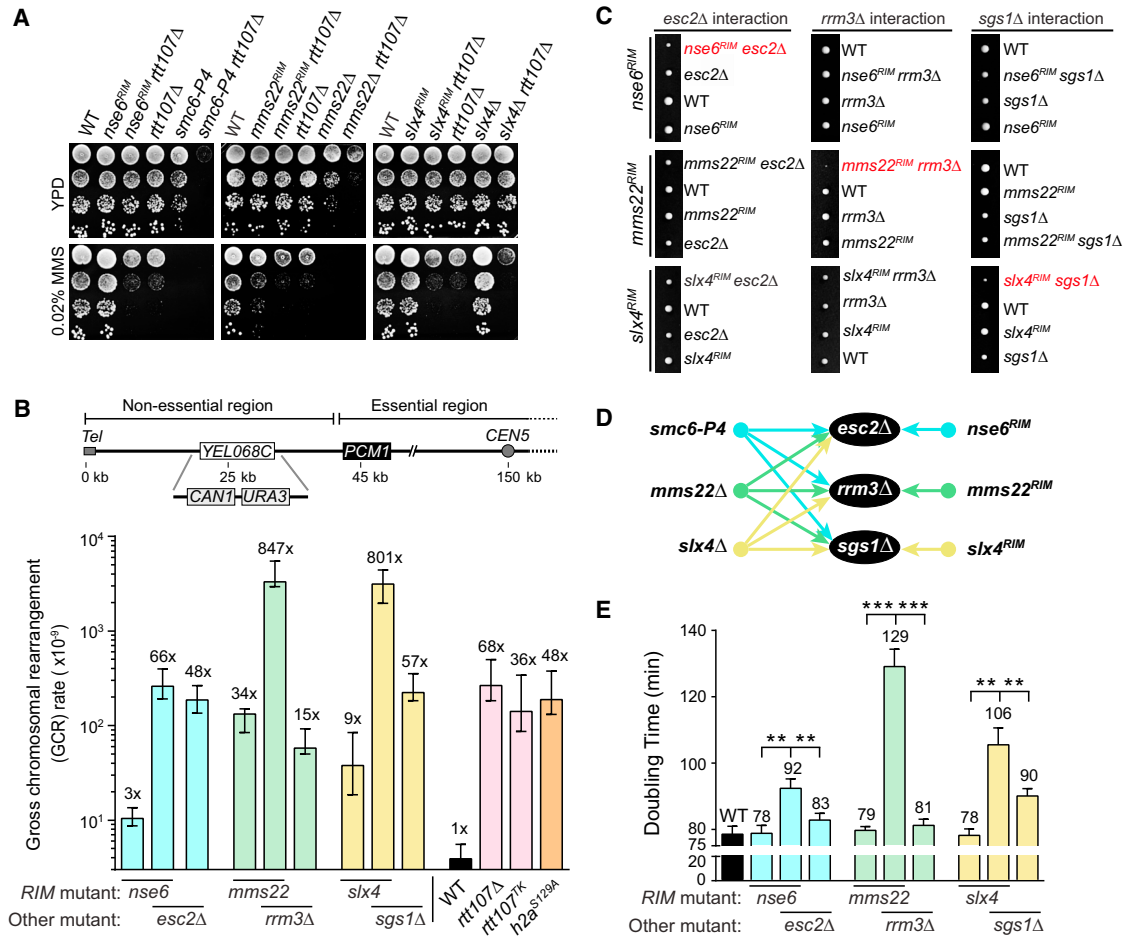


Figure 5. Rtt107 Binding of Partner Proteins Promotes Genomic Stability during Normal Growth

(A) The *Rtt107* mutants of *nse6*, *mms22*, and *slx4*, unlike their null or hypomorphic mutants, are epistatic with *rtt107Δ* during growth (YPD) and under damage conditions (MMS [methyl methanesulfonate]). Cells were spotted in 10-fold serial dilutions.

(B) The *Rtt107* mutants, *rtt107* mutants, and *h2a^{S129A}* cells exhibit elevated GCR rates. For each genotype, median rate of at least nine cultures was calculated from two biological duplicates. Error bars are the 95% confidence intervals. Schematic of the GCR assay is shown in the top panel.

(C) Representative tetrads of diploids heterozygous for indicated mutations. Negative genetic interactions are colored red.

(D) Summary of the genetic interactions of *Rtt107* mutants and their corresponding null or hypomorphic alleles with tester strains. Arrows indicate synthetically sick or lethal interactions.

(E) Cell doubling-time measurement confirmed the negative genetic interactions between specific *Rtt107* mutants and deletions of distinct buffering genes. Three biological duplicates were used for calculation. Averages and SDs are shown; **p < 0.01 and ***p < 0.001.

See also Figure S6.

Different *Rtt107* Mutants Are Synthetically Sick with Unique Genome Stability Mutants

To further understand how *Rtt107* mutants affect genomic stability, we asked whether they had unique genetic interactor(s). A targeted screen among mutants reported to show negative genetic interactions with *rtt107Δ* or mutants of the Smc5/6 complex, Mms22, and Slx4, found that each *Rtt107* mutant was synthetically sick with the loss of a specific genome stability factor (Figures 5C and 5D). These include the Sgs1 and Rrm3 DNA helicases and the Esc2 structural protein, which affect different aspects of the DNA replication and repair processes. In contrast to *Rtt107* mutants, the null or hypomorphic alleles of Mms22, Slx4, or the Smc5/6 complex showed negative interactions with all three tester mutants (Figures S6B–S6D). The *Rtt107* genetic interactions

were also verified by cell doubling-time measurements (Figure 5E). In GCR tests, the double mutants of *nse6^{RIM} esc2Δ*, *mms22^{RIM} rrm3Δ*, and *slx4^{RIM} sgs1Δ* exhibited a further increase in the rates of *Rtt107* mutants, up to ~850-fold over wild-type levels (Figure 5B). These findings suggest that the functions of Rtt107 binding to distinct client proteins are related to their unique genetic interactors.

Rtt107 Binding of Client Proteins Supports Chromosome Replication and rDNA Stability

Increased GCR levels and genetic interactions of *Rtt107* mutants described above can both be caused by defective DNA replication (Putnam et al., 2009). We thus tested whether *Rtt107* mutants impair the duplication of chromosome 12, which provides a

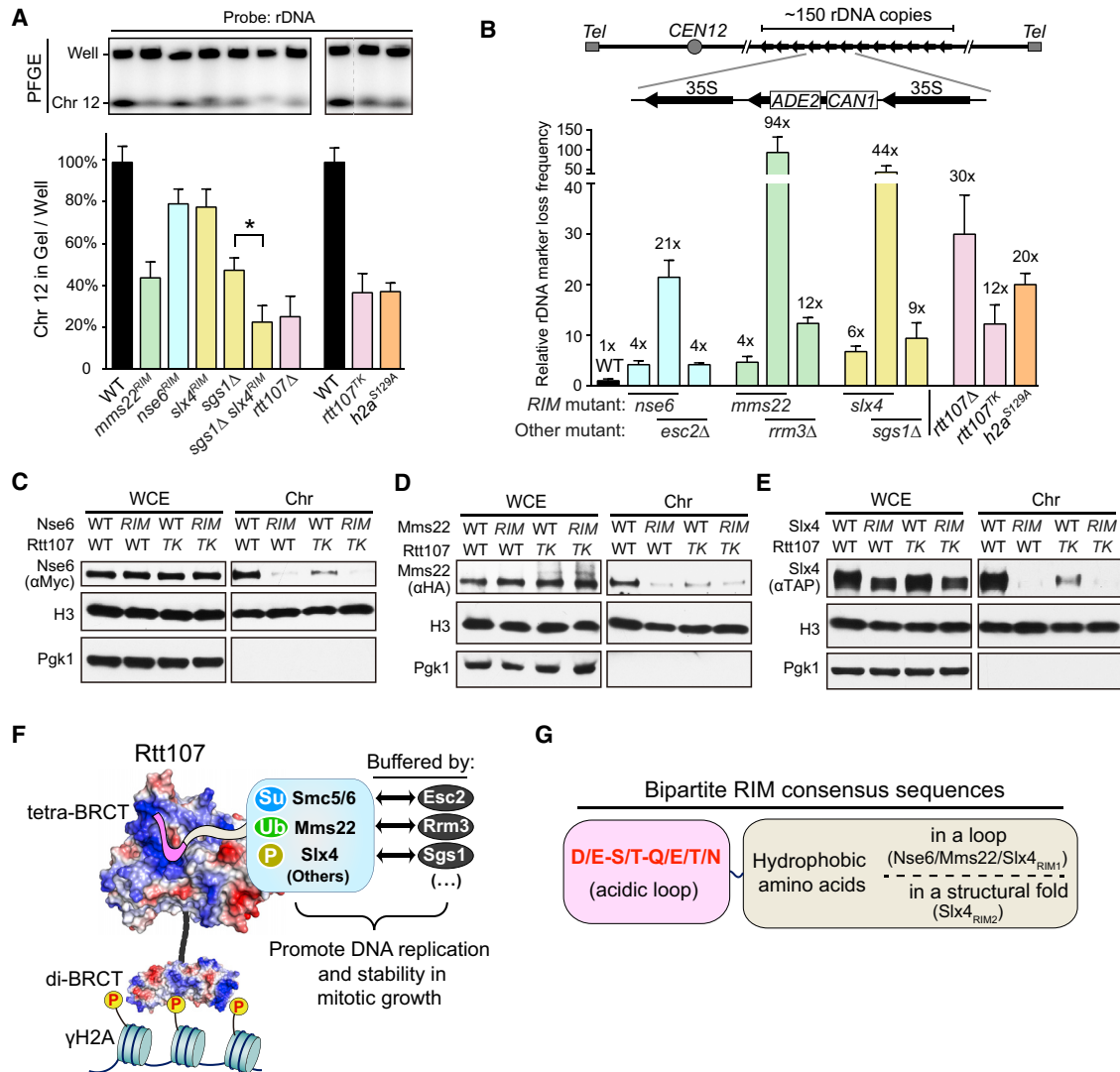


Figure 6. Rtt107 Targets Partner Proteins to Chromatin to Promote Genome Stability during Growth

(A) Examination of chromosome 12 replication. Southern blots were quantified to derive chromosome signals in gel versus well. Values were normalized to wild-type. Averages and SDs of two biological duplicates are indicated. Differences between mutants and wild-type, as well as between the two indicated mutants, were statistically significant ($p < 0.05$).

(B) rDNA marker loss rate measurement. Top: schematic of rDNA marker loss assay as described previously (Fritze et al., 1997). Bottom: averages of marker loss rates and SDs were calculated from three biological duplicates.

(C–E) Chromatin association of Rtt107 partner proteins. Whole-cell extract (WCE) and chromatin fraction (Chr) of Nse6 (C), Mms22 (D), and Slx4 (E) were examined using western blotting. H3 and Pgk1 are as markers for chromatin and non-chromatin fractions, respectively.

(F) A structural and functional model for Rtt107 control of diverse genome factors and pathways. Protein modification functions of Rtt107 partner proteins are highlighted: Su, the SUMO E3 function of the Smc5/6 complex; Ub, ubiquitin E3 function of the Mms22 Cul8 complex; P, a role of Slx4 in dampening checkpoint kinase phosphorylation.

(G) Bi-partite Rtt107 tetra-BRCT interaction consensus sequences derived from this study.

See also Figure S7.

sensitive indicator for DNA replication defects because of its containing of the difficult-to-replicate rDNA. We used pulsed-field gel electrophoresis (PFGE), which allows fully replicated chromosomes to enter the gel but traps partially replicated chromosomes in the gel wells. We found that *nse6^{RIM}*, *mms22^{RIM}*, and *slx4^{RIM}* mutants were each defective in replicating chromosome 12 in S phase, and that *rtt107^Δ* cells ex-

hibited a more severe defect, likely reflecting the combined loss of its interactions (Figure 6A). Moreover, deleting the *SGS1* gene, which can buffer *slx4^{RIM}* growth defect and GCR increase, exacerbated the chromosome 12 replication defect in *slx4^{RIM}* cells (Figure 6A). These results indicate that Rtt107 tetra-BRCT interactions with Nse6, Mms22, and Slx4 promote chromosome replication.

We extended this analysis by examining the stability of the rDNA region on chromosome 12. The loss rates of an *ADE2-CAN1* cassette inserted in the rDNA array increased several-fold in *nse6^{RIM}*, *mms22^{RIM}*, or *slx4^{RIM}* mutants compared with wild-type cells (Figure 6B). Moreover, these defects were enhanced when the buffering gene for each *RIM* mutant was deleted, such that combined mutants exhibited up to ~100-fold increase of rDNA marker loss over wild-type (Figure 6B). Thus, these RIM-dependent Rtt107 interactions protect rDNA stability.

Rtt107 Tetra- and Di-BRCT Domains Cooperatively Target Client Proteins to Chromatin

Our data so far suggest that though the different Rtt107 interactions have distinct roles, they all contribute to genome replication and stability during growth. This raises the possibility of a unified theme underpinning their genomic effects. Because the Rtt107 BRCT5 and BRCT6 recognize γ H2A, which marks replicating chromatin during growth (Li et al., 2012; Szilard et al., 2010), we hypothesized that the Rtt107 tetra-BRCT domain might collaborate with this di-BRCT to recruit the various interacting proteins to chromatin. This model predicts that disrupting the interactions of tetra-BRCT and client proteins or that of di-BRCT and γ H2A should both reduce chromatin-bound client protein levels. This was indeed the case for *Nse6^{RIM}*, *Mms22^{RIM}*, and *Slx4^{RIM}* mutant proteins (Figures 6C–6E). In addition, a γ H2A binding mutant, *rtt107^{TK}* (T842A/K887M) (Li et al., 2012), greatly reduced the levels of chromatin-bound *Nse6*, *Mms22*, and *Slx4* (Figures 6C–6E). Moreover, the effects of *RIM* mutants were epistatic with *rtt107^{TK}* (Figures 6C–6E). These results suggest that Rtt107 serves as a molecular bridge to connect multiple tetra-BRCT-binding proteins with chromatin.

Rtt107-Mediated Chromatin Targeting of Client Proteins Aids Genome Stability and Replication

We hypothesized that the bridging function of Rtt107 relying on both its di- and tetra-BRCT domains is important for genome maintenance. This model predicts that *rtt107^{TK}* and *h2a^{S129A}* (Szilard et al., 2010), which abrogate Rtt107 di-BRCT- γ H2A interaction while retaining its tetra-BRCT-RIM interactions, should exhibit similar or stronger defects compared with *RIM* mutants. Indeed, GCR and rDNA marker loss assays and chromosomal replication data supported this prediction (Figures 5B, 6A, and 6B). These findings strengthen our model and suggest that Rtt107-mediated targeting of client proteins to chromatin is critical for genome replication and stability.

DISCUSSION

BRCT domain-mediated protein interactions are integral to the genome maintenance network. Our structural, biochemical, and mutagenesis data demonstrate that the compact Rtt107 tetra-BRCT structure recognizes a variety of unmodified peptides through a common bi-partite binding strategy. Our *in vivo* findings identified housekeeping roles of multiple Rtt107 interactions in genome maintenance and a concerted action of two types of BRCT domains in Rtt107 in targeting client proteins to chromatin during growth. On the basis of these insights into

Rtt107 tetra-BRCT structure, its client recognition mechanisms, and its collaboration with di-BRCT in controlling client proteins and genome maintenance, we propose a mechanistic model of how the Rtt107 master regulator regulates multiple genome functions by serving as a molecular bridge (Figure 6F and details below).

A Higher-Order Tetra-BRCT Structure and Its Client Recognition Mechanism

Our study shows that the Rtt107 tetra-BRCT structure is not simply a combined pair of di-BRCTs. The ability of multiple BRCT repeats to fold into a tetrahedral assembly rather than a linear or planar form suggests that additional “BRCT origami” may be possible. This finding underlines the importance of examining the entire region containing multiple BRCT domains rather than subsets of its repeats. Our data also show that the unique tetra-BRCT structure enables a different ligand recognition mechanism than that of di-BRCTs (more below). This first example (to our knowledge) of domain repeats forming a highly compact assembly to acquire new functions may be applicable to other repetitive domains generated by gene duplication during evolution.

Unlike classical di-BRCT domains that recognize phospho-Ser/Thr peptides, we showed that Rtt107 tetra-BRCT bound to multiple unphosphorylated ligands. This finding expands our perception of BRCT domain functions. Our data further demonstrate that Rtt107 tetra-BRCT uses a bi-partite mechanism in client recognition, wherein RBIP recognizes the D/E-S/T-Q/E/T/N motif of the RIMs and a hydrophobic groove engages a hydrophobic region of RIMs (Figure 6G). We also found variations under this scheme. For example, while the electrostatic interface is more critical for Rtt107 interactions with *Nse6* and *Slx4*, the Rtt107-*Mms22* interaction also heavily relies on their hydrophobic interface. We found that Rtt107_{NTD} interactions with additional RIMs from *Scm3*, *Rad55*, and *Cdc7* were similar to those with *Nse6* and *Slx4*. It is thus likely that Rtt107 tetra-BRCT recognizes the D/E-S/T-N/Q/E/T motif and relies on the hydrophobic interface to fine-tune affinity in order to accommodate large numbers of ligands.

We suggest that the shared interaction mechanism of multiple Rtt107 client proteins ensures a binary interaction between Rtt107 and proteins of different activities, thus preventing promiscuity among different functions. As the two *Slx4* RIMs use a similar mechanism to bind Rtt107 and show competition in Rtt107 association, they likely contribute to different *Slx4*-Rtt107 complexes. The specific mode by which *Slx4* engages with Rtt107 may determine how they associate with additional proteins that have been previously identified (Gritenaite et al., 2014; Ohouo et al., 2010; Princz et al., 2017). Rtt107 could also use additional surfaces and protein modifications for regulating binding and increasing the plasticity of its interactomes. Indeed, Rtt107 has been shown to be phosphorylated (Rouse, 2004), and the RIM sequences contain consensus sites for CDK, DDK, and Mec1 kinases. As such, the Rtt107 interactome may be subject to protein modification-based regulation. Further exploration of these topics will expand our understanding of the scope of the Rtt107 interactome and its regulation in the future.

Unique and Concerted Genomic Effects of the Rtt107 Interactions with Client Proteins

Our analyses of *RIM* alleles that specifically disrupt the Rtt107 interactions with Nse6, Mms22, or Slx4 reveal that these interactions constitutively support genome stability during growth, altering the view that BRCT domains contribute mainly to the DNA damage response. We found that Rtt107 interactions with Nse6, Mms22, or Slx4 all contribute to the maintenance of rDNA, a site highly prone to DNA fragility and instability. As rDNA also influences genomic stability and RNA and protein synthesis by organizing nucleoli, the Rtt107 interactome can link DNA stability to overall cellular fitness.

Our data further suggest that each examined Rtt107 interaction has unique effects, because the three *RIM* mutants render different levels of genome instability and their phenotype were buffered by distinct genes. We linked these unique effects to specific genome maintenance pathways mediated by Sgs1, Rrm3, or Esc2, and suggest that they likely extend beyond previously suggested functions under damage conditions, such as DNA damage checkpoint regulation or large replicon synthesis (Ohouo et al., 2013; Hang et al., 2015). Because Rtt107 client proteins affect a large spectrum of genome maintenance functions, including DNA replication, repair, and segregation, the Rtt107-based network has a broad influence on genome fitness. The *RIM* mutants will provide tools for further mechanistic studies of the entire spectrum of functions of this network.

Collaboration of Rtt107 Tetra- and Di-BRCT Domains in Chromatin Targeting of Client Proteins

We found that the Rtt107 tetra-BRCT domain acts in concert with its di-BRCT domain to target client proteins to chromatin, supporting a model in which Rtt107 controls multiple genomic stability factors by targeting them to γ H2A-containing chromatin during growth. Our findings unify and extend previous proposals made under genotoxic conditions for damage site localization (Balint et al., 2015; Leung et al., 2011). Importantly, the present work provides a structural and mechanistic basis for these effects and demonstrates that a chromatin recruitment function of Rtt107 safeguards the genome during cycles of mitotic growth. Our findings also underline the importance of studying the integrated functions of different sets of BRCT domains within the same protein.

Because γ H2A can spread over several kilobases during growth (Ström et al., 2007; Szilard et al., 2010), Rtt107 likely affects vast tracts of the genome. Additionally, its tetra-BRCT interactors possess diverse activities allowing Rtt107 to orchestrate diverse functions. We further envision that Rtt107 complements the other hub proteins to generate a repertoire of mechanisms that deliver different sets of factors to specific DNA and chromatin regions (Figure S7A). Together, they may be part of a nuclear protein sorting system that recognizes specific peptides as zip-code sequences to enable localization to unique genomic regions. These mechanisms may complement the general protein sorting system to fine-tune DNA metabolism protein localization.

As seen for Rtt107, the BRCT1–4 domains of the mammalian PTIP and fission yeast Brc1 also lack phospho-peptide binding sites, while their BRCT5 and BRCT6 domains bind to γ H2A (Li

et al., 2012; Manke et al., 2003; Williams et al., 2010; Yan et al., 2011; Yu et al., 2003). We thus speculate that PTIP and Brc1 may act in a similar fashion to Rtt107, using their BRCT1–4 domains to recognize unphosphorylated client proteins and target them to DNA replication and damage sites. Although the tetra-BRCT fold is difficult to predict at the sequence level, RIM-like sequences are found in Nse6, Mms22, and Slx4 homologs from species closely related to budding yeast and in fission yeast, which has a large evolutionary distance from budding yeast, suggesting the conservation of this sequence (Figures S7B and S7C). Thus, our findings can stimulate understanding of how multi-BRCT proteins recognize specific client proteins and control complex genome metabolism processes in other organisms.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- DATA AND CODE AVAILABILITY

SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

B.W. obtained protein crystals and X-ray diffraction data and performed biochemical and *in vivo* tests. J.W. processed diffraction data to derive

structural models. X.M. performed the PFGE experiments. M.L. oversaw structure quality control and described structural results. X.Z. supervised the overall study, analyzed and interpreted the data, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-HA mouse monoclonal IgG2a (Clone: F-7)	Santa Cruz Biotech	Cat# sc-7392, RRID: AB_627809
Anti-Myc mouse monoclonal IgG1 (Clone: 9E10)	Bio X Cell	Cat# BE0238, RRID: AB_2687720
Anti-Flag mouse monoclonal IgG1 (Clone: M2)	Sigma-Aldrich	Cat# F1804, RRID: AB_262044
Anti-TAP rabbit antibody	Sigma-Aldrich	Cat# P1291, RRID: AB_1079562
Anti-Pgk1 mouse monoclonal IgG1 (Clone: 22C5D8)	Invitrogen	Cat# 459250, RRID: AB_2532235
Anti-Histone H3 rabbit antibody	Abcam	Cat# ab46765, RRID: AB_880439
Anti-mouse HRP-linked antibody	GE Healthcare	Cat# NA 931V, RRID: AB_772210
Anti-rabbit HRP-linked antibody	GE Healthcare	Cat# NA 934V, RRID: AB_772206
Bacterial Strains		
TOP10 Chemically Competent Cells	Invitrogen	Cat# C4040-10
BL21(DE3) Competent Cells - Novagen	EMD Millipore	Cat# 69450-4
Chemicals, Peptides, and Recombinant Proteins		
2-nitrophenyl β -D-galactopyranoside (ONPG)	Sigma-Aldrich	Cat# N1127-5G
ULP1 Protease	Home-made	N/A
PreScission (3C) Protease	GE Healthcare	Cat# 27084301
Deposited Data		
Structure of Rtt107 _{NTD} (tetra-BRCT)	This paper	PDB: 6J0V
Structure of Rtt107 _{NTD} -Nse6 _{RIM}	This paper	PDB: 6J0W
Structure of Rtt107 _{NTD} -Mms22 _{RIM}	This paper	PDB: 6J0X
Structure of Rtt107 _{NTD} -Slx4 _{RIM2}	This paper	PDB: 6J0Y
Mendeley Dataset	This paper	http://dx.doi.org/10.17632/bj532s6vd4.2
Recombinant DNA		
pRS315-YEL072W::URA3/CAN1 (for W303 GCR strain)	R. Kolodner	pRDK1378
pRS315-YEL068C::URA3/CAN1 (for W303 GCR strain)	R. Kolodner	pRDK1379
pFA6a-4GS-6HA::NAT (for yeast gene tagging PCR)	This paper	pXZ1043
pBTM116-Rtt107 _{NTD} (2-513a.a.)	This paper	pXZ991
pGADT7-Nse6-FL	Zhao Lab	pXZ459
pACT2-Nse6 _{RIM} (2-46 a.a.)	This paper	pXZ990
pACT2-Mms22-FL	This paper	pXZ987
pACT2-Mms22 _{RIM} (2-38 a.a.)	This paper	pXZ986
pGADT7-Slx4-FL	Zhao Lab	pXZ458
pACT2-Slx4 _{RIM1} (407-445 a.a.)	This paper	pXZ989
pACT2-Slx4 _{RIM2} (535-587 a.a.)	This paper	pXZ988
pACT2-Scm3-FL	This paper	pXZ985
pACT2-Scm3 _{RIM} (165-223 a.a.)	This paper	pXZ984
pACT2-Rad55 _{RIM} (371-406 a.a.)	This paper	pXZ983
pACT2-Cdc7-FL	This paper	pXZ981
pACT2-Cdc7 _{RIM} (470-507 a.a.)	This paper	pXZ982
pGEX-6P-1-Rtt107 _{NTD} (codon-optimized Rtt107 ₂₋₅₁₃)	This paper	pXZ968
pGEX-6P-1-Nse6 _{RIM} (for peptide expression in <i>E. coli</i>)	This paper	pXZ967
pET28a-6His-Sumo-Mms22 _{RIM} (expression in <i>E. coli</i>)	This paper	pXZ966
pET28a-6His-Sumo-Slx4 _{RIM1}	This paper	pXZ965
pGEX-6P-1-Slx4 _{RIM1}	This paper	pXZ960
pGEX-6P-1-Slx4 _{RIM2}	This paper	pXZ964

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pGEX-6P-1- Scm3 _{RIM}	This paper	pXZ963
pGEX-6P-1- Rad55 _{RIM}	This paper	pXZ962
pGEX-6P-1- Cdc7 _{RIM}	This paper	pXZ961
Experimental Models		
Organisms/Strains (<i>S. cerevisiae</i> /W303)	See Table S1	N/A
Oligonucleotides		
Nse6 ^{RIM} -mut-FP: GAAACTGTACCAGCCGCGGCGATTTCAGGGTTTG	IDT	N/A
Slx4 ^{RIM1} -mut-FP: CTATTGTCTCAGATGCGGCTGATGAGACATCCAC	IDT	N/A
Slx4 ^{RIM2} -mut-FP: GTTGAGGCAATGATGCGGAAGAAGAGGAGACA	IDT	N/A
Mms22 ^{D13A} -mut-FP: GTGATATCAGCGTCTGAGGCCACTG	IDT	N/A
Mms22 ^{Y33A} -mut-FP: CGAATTC AATGAAAATGCTTTATGGGCAGAGG	IDT	N/A
Software and Algorithms		
HKL3000	Minor	https://www.hkl-xray.com
Phenix	Adams	https://www.phenix-online.org
Coot	Emsley	https://www2.mrc-lmb.cam.ac.uk/Personal/pemsley/coot/
PyMOL	Schrodinger, LLC	https://pymol.org/2/
Origin 7	OriginLab	N/A
ImageJ	ImageJ software	N/A
GraphPad Prism 7	GraphPad Software	N/A
Others		
Ni-NTA Agarose	QIAGEN	Cat# 30230
Glutathione Sepharose 4B beads	GE Healthcare	Cat# 17-0756-05

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by, the Lead Contact, Xiaolan Zhao (zhaox1@mskcc.org).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Yeast strains are listed in the Key Resources Table.

METHOD DETAILS

Protein expression and purification

Codon-optimized Rtt107_{NTD} (residues 2-513) was fused to a GST tag and expressed from the pGEX-6P-1 vector in the BL21 (DE3) *Escherichia coli* strain. Cells grown at 37°C in 12 L TB (terrific broth) media were induced for protein expression with 0.2 mM IPTG for 16 h at 20°C. Cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 400 mM NaCl, 10% glycerol, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 5 mM benzamide, and 4 mM β-mercaptoethanol) and sonicated. After ultracentrifugation to remove cell debris, supernatant was incubated with glutathione Sepharose 4B beads for 2 h at 4°C. Beads were washed with lysis buffer and the Rtt107_{NTD} protein was eluted with the addition of 15 mM reduced glutathione (Sigma). Following the removal of the GST tag by the PreScission protease, ion exchange (Mono Q column) and gel-filtration chromatography (Superdex 200 column) were used to purify untagged Rtt107_{NTD} to close to homogeneity. Peak fractions containing Rtt107_{NTD} were concentrated and stored at –80°C.

The GST tag was fused to Nse6_{RIM} (residues 2-46), Slx4_{RIM1} (residues 407-445), Slx4_{RIM2} (residues 535-587), Scm3_{RIM} (residues 165-223), Rad55_{RIM} (residues 371-406), and Cdc7_{RIM} (residues 470-507) peptides and the fusions proteins were expressed from the pGEX-6P-1 vector in the BL21 (DE3) strain. Cells grown at 37°C in 2 L LB media were induced for protein expression with 0.1 mM IPTG for 12 h at 24°C. The expressed proteins were first purified by binding to glutathione Sepharose 4B beads. For GST, Slx4_{RIM1}-GST, and Slx4_{RIM2}-GST, the proteins were further purified by gel-filtration chromatography (HiLoad 16/600 Superdex 200). For the other fusion proteins, GST tag was cleaved by the PreScission protease and the peptides were further purified by gel-filtration chromatography (HiLoad 16/600 Superdex 75).

6xHis-SUMO tagged Mms22_{RIM} (residues 2-38) and Slx4_{RIM1} (residues 407-445) peptides were expressed from the pET-28a vector and purified as described above, except that Ni-NTA agarose beads (QIAGEN) were used for affinity purification and 10 mM imidazole was included in the lysis buffer. The tag was removed by the Ulp1 protease and the Mms22_{RIM} or the Slx4_{RIM1} peptide was further purified by gel-filtration chromatography (HiLoad 16/600 Superdex 75). Glutathione beads, chromatography columns, and proteases used in this study were purchased from GE Healthcare.

Crystallization, data collection, and structure determination

Crystals of purified Rtt107_{NTD} were grown by sitting-drop vapor diffusion. The precipitant well solution consisted of 1.8 M (NH₄)₂SO₄, 50 mM pH 5.8 sodium cacodylate, 5 mM Mg(OAc)₂, and 0.5 mM spermine. Crystals were gradually transferred into a harvesting solution containing 2 M (NH₄)₂SO₄, 50 mM pH 5.8 sodium cacodylate, 15 mM Mg(OAc)₂, 1 mM spermine, and 25% glycerol, followed by flash-freezing in liquid nitrogen for storage. For preparation of a mercury derivative, ethyl mercuric phosphate was soaked into the Rtt107_{NTD} crystals for 1 h at 4°C.

The Rtt107_{NTD} protein was mixed with each ligand peptide at a molar ratio 1:2 to form the corresponding complex. The Rtt107_{NTD}-Nse6_{RIM} complex crystals were grown by sitting-drop vapor diffusion with a well solution containing 22% PEG 3350, 0.1 M pH 6.0 Bis-Tris, and 0.2 M NH₄OAc. For the Rtt107_{NTD}-Slx4_{RIM2} complex, the well solution contained 10% PEG 6000, 0.1 M pH 7.0 HEPES, and silver bullets additive G9 (Hampton Research). For the Rtt107_{NTD}-Mms22_{RIM} complex, the well solution contained 25% PEG 3350, 0.1 M pH 6.0 Bis-Tris, and 0.2 M MgCl₂. All crystals were gradually transferred into harvesting solution and flash-frozen in liquid nitrogen for storage.

Datasets were collected under cryogenic conditions (100K) at Advanced Photon Source (APS) beamline 21ID-D and 24ID-C. A 2.8 Å Hg-SAD (single-wavelength anomalous dispersion) dataset of Rtt107_{NTD} was collected at the wavelength of 0.97623 Å and was processed by HKL3000 (Minor et al., 2006). Three mercury atoms were located and refined, and the single-wavelength anomalous diffraction data phases were calculated using Phenix (Adams et al., 2010). The initial SAD map was substantially improved by solvent flattening. An initial model was automatically built into the modified experiment electron density. The model was then refined with a native dataset with a 2.3 Å resolution with manual building in Coot (Emsley et al., 2010). The Rtt107_{NTD}-Nse6_{RIM}, Rtt107_{NTD}-Slx4_{RIM2} and Rtt107_{NTD}-Mms22_{RIM} complex structures were solved by molecular replacement with the Rtt107_{NTD} native structure as the searching model. The models were refined using Phenix, together with manual building in Coot. All the structural figures were generated using PyMOL (<https://pymol.org/2/>).

Isothermal titration calorimetry

Dissociation constants of the Rtt107_{NTD} interactions with ligand peptides were determined using an iTC200 calorimeter (MicroCal). Binding enthalpies were monitored when peptides were injected into cells containing Rtt107_{NTD} in 25 mM pH 8.0 Tris-HCl and 150 mM NaCl at 16°C. As controls, peptides were injected into buffer lacking Rtt107_{NTD}. Three independent experiments were performed for each interaction, and data were calculated and fitted using Origin 7 software (OriginLab).

Yeast strain construction and genetic manipulation

Yeast strains are derivatives of W1588-4C, a *RAD5* variety of W303 (*MATa ade2-1 can1-100 ura3-1 his3-11,15 leu2-3,112 trp1-1 rad5-535*) (Zhao et al., 1998). Strains are listed in Table S1. All proteins were expressed from their endogenous loci. Protein tagging, gene deletion, and point mutation were generated following standard PCR-based or CRISPR-Cas9 methods (DiCarlo et al., 2013). A GCR assay strain in the W303 background was constructed following a previously described procedure (Putnam et al., 2009). Briefly, the endogenous *URA3* ORF (Chr 5: 116167-116970) and *CAN1* ORF (Chr 5: 31694-33466) were deleted by CRISPR-Cas9 method to prevent assay interference. To insert the *URA3-CAN1* cassette at the *YEL068C* locus (Chr 5: 25646-25978), the pRDK1379 plasmid (KEY RESOURCES TABLE) was digested with PvuII (NEB) and transformed into yeast cells. We verified that GCR rates for wild-type and *sgs1Δ* strains were similar to those reported previously (Putnam et al., 2009).

All genetically altered loci were verified by sequencing. Standard yeast genetic manipulation was used for tetrad analyses and spotting assays. Pictures were taken after plates were incubated for 2 days at 30°C. To determine doubling time, OD₆₀₀ of yeast cultures grown in YPD media were assessed every 10 min using SpectraMax M5 microplate spectrophotometer. OD₆₀₀ values from early-mid log phase were used to calculate doubling time (T_D) using the formula: T_D = ln(2/k). k (constant of growth) = (ln(N_x) - ln(N₀))/(T_x-T₀); N₀ and N_x represent OD₆₀₀ values at time points T₀ and T_x (in minutes), respectively.

Yeast two-hybrid assay

The yeast two-hybrid assays were performed as previously described (Wan et al., 2015). Briefly, Rtt107_{NTD}, as well as various fragments of Nse6, Slx4, Mms22, Scm3, Rad55, and Cdc7 was cloned into pBTM116 (BD) and pACT2 (AD) vectors (Clontech) at the BamHI and XhoI sites (KEY RESOURCES TABLE). Mutations were made by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene). Yeast cells harboring BD and AD plasmids were selected on SC-Leu-Trp plates. β-galactosidase activities were measured according to Clontech Matchmaker yeast two-hybrid protocol with o-nitrophenyl β-D-galactopyranoside (ONPG) as substrate. The averages from three transformants were calculated and error bars represent standard deviations.

Co-immunoprecipitation

Log-phase cells were harvested and resuspended in IP buffer (20 mM pH 8.0 HEPES-KOH, 150 mM KOAc, 2 mM Mg(OAc)₂, 10% glycerol, 5 μM leupeptin, 1 mM PMSF, 1 mM NaF, 0.5 mM Na₃VO₄, 2 mM β-glycerophosphate, Sigma protease inhibitor cocktail, 0.6% Triton X-100), followed by lysis with glass beads in a FastPrep-24 homogenizer (MP Biomedicals). Benzonase (EMD Millipore) was added to degrade DNA. Supernatant after centrifugation was incubated with IgG-Sepharose (binds TAP), anti-FLAG, or anti-HA beads (Sigma) for 2 h at 4°C. After washing beads with IP buffer, proteins were eluted with loading buffer (125 mM pH 6.8 Tris-HCl, 4% SDS, 20% glycerol, 0.02% bromophenol blue) at 85°C for 5 min. DTT was added to protein samples before assaying by SDS-PAGE on 4%–20% gels (Bio-Rad). Antibodies used in western blotting include anti-HA (Santa Cruz Biotech), anti-Myc (Bio X Cell), anti-Flag (Sigma), anti-TAP (Sigma).

PFGE analysis

G1-arrested cells were released to S phase for 45 min and embedded into agarose plugs for PFGE analyses as previously described (Cremona et al., 2012). Briefly, plugs were treated with zymolyase (20T, MP Biomedicals), proteinase K, and lauroylsarcosine to permeabilize cells. Chromosomes were separated by 1% agarose (Bio-Rad) gels in 0.5 × TBE buffer using the Bio-Rad CHEF-DR III PFGE system. Gel running conditions were 70–160 s switch time, 5.5 V/cm voltage gradient, and 106° angle for 15 h at 12°C. After electrophoresis, chromosomes were transferred to Hybond-XL nylon membranes (GE) for Southern blotting using an [α -³²P]-dCTP-labeled rDNA probe. Autoradiographic signals were scanned by Typhoon FLA 9500 phosphorimager (GE), and rDNA replication efficiency was assessed by calculating the ratio of chromosome band signals to the corresponding well signals after adjusting for background signals. Quantification of chromosomal bands was performed using the ImageJ software.

GCR rate measurement

We determined GCR rates in W303 strain background according to fluctuation analysis described previously (Putnam and Kolodner, 2010). For each genotype, at least 9 cultures were examined in at least two different strains. Yeast cells were washed and serial dilutions were plated on SC+5-FOA+Can (FC) and SC plates. Cells which lose the *CAN1-URA3* cassette are resistant to canavanine and 5-FOA. After inoculation of plates for 3–4 days at 30°C, colonies on FC and SC plates were counted. GCR rates (R_G) were calculated by the following equation: $R_G = m/N_T$, wherein $m(1.24 + \ln[m]) - N_{FC} = 0$. m : mutational events, N_{FC} : number of colonies on FC plates, N_T : number of total cells spread on the FC plates, which was deduced from the number of colonies on SC plates. The upper and lower 95% confidence intervals (95% CI) were calculated as described (Putnam and Kolodner, 2010).

rDNA marker loss frequency

The loss of the *ADE2-CAN1* cassette inside the rDNA array was examined as previously described (Fritze et al., 1997). Briefly, cells were grown for equal doublings to stationary phase. Cells were plated on SC media for total cell counts. Cells were also plated on canavanine-containing media (SC+Can), then replica plated to media lacking adenine (SC-Ade). Marker loss frequency (F_R) was calculated as described previously (Bernstein et al., 2011), using the following formula: $F_R = (N_{Can} - N_{Ade})/N_C$, wherein N_{Can} = number of colonies on SC+Can plates, N_{Ade} = number of colonies on SC-Ade plates, and N_C = number of cells plated on SC+Can plates. Marker loss frequencies of mutant strains were normalized to wide-type.

Chromatin fractionation

Chromatin fractionation was performed as described previously (Chung and Zhao, 2015). In brief, spheroplasts from log-phase cells were lysed using extraction buffer (20 mM pH 6.6 PIPES-KOH, 150 mM KOAc, 2 mM Mg(OAc)₂, 1 mM NaF, 0.5 mM Na₃VO₄, 1 × Sigma protease inhibitors, 1% Triton X-100) for 5 min on ice. After centrifugation at 16,000 g for 15 min on a sucrose cushion, chromatin pellets were washed and resuspended with extraction buffer. Protein loading buffer was added to cell extract and chromatin fraction and boiled for 5 min before SDS-PAGE followed by western blotting. Pgk1 and histone H3 are markers for non-chromatin and chromatin associated proteins, respectively. Antibodies used are as described above, except that anti-Pgk1 (Invitrogen) and anti-H3 (Abcam) were also used.

In vitro competition assay

100 μg Slx4_{RIM2}-GST or GST protein and 60 μg Rtt107_{NTD} were incubated in 50 μL binding buffer (25 mM pH 8.0 Tris-HCl, 150 mM NaCl and 1 mM DTT) for 15 min at 4°C. Mms22_{RIM}, Slx4_{RIM1} or Nse6_{RIM} used added into the complex in a ratio (Rtt107_{NTD}: Slx4_{RIM2}-GST: competitor = 1: 2: 4 in Figure 4G) to compete for 15 min. The mixture was incubated with 10 μL of glutathione agarose resin to retain Slx4_{RIM2}-GST or GST for 1 h. After washing the resin five times with 100 μL of binding buffer, bound proteins were eluted with 20 μL SDS protein sample buffer. 2 μL eluted samples and input samples (5% protein of competition assay) were analyzed by 4%–20% gradient SDS-PAGE and Coomassie blue staining. Similar procedure was used for Figure 4H, except that peptides and their concentrations varied as indicated in the graph. Quantification of protein bands was performed using the ImageJ software.

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification of PFGE data (Figure 6A) and protein bands on gels (Figures S5B and S5C) was performed using the ImageJ software. Statistical analyses were performed using the GraphPad Prism 7 software, except that the ITC data were analyzed using the Origin 7 software. Additional details of statistical analyses are described in Figure Legends and in Results. Data are represented as mean \pm standard deviation, except that for the GCR assay data, error bars are the 95% confidence intervals.

DATA AND CODE AVAILABILITY

The following coordinates have been deposited in the RCSB Protein Data Bank: apo-Rtt107_{NTD} (PDB: 6J0V); Rtt107_{NTD}-Nse6_{RIM} complex (PDB: 6J0W); Rtt107_{NTD}-Mms22_{RIM} complex (PDB: 6J0X); Rtt107_{NTD}-Slx4_{RIM2} complex (PDB: 6J0Y).