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The Sir4 H-BRCT domain interacts with phospho-proteins to sequester and repress yeast heterochromatin

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Abstract

In *Saccharomyces cerevisiae*, the silent information regulator (SIR) proteins Sir2/3/4 form a complex that suppresses transcription in subtelomeric regions and at the homothallic mating-type (HM) loci. Here, we identify a non-canonical BRCA1 C-terminal domain (H-BRCT) in Sir4, which is responsible for tethering telomeres to the nuclear periphery. We show that Sir4 H-BRCT and the closely related Dbf4 H-BRCT serve as selective phospho-epitope recognition domains that bind to a variety of phosphorylated target peptides. We present detailed structural information about the binding mode of established Sir4 interactors (Esc1, Ty5, Ubp10) and identify several novel interactors of Sir4 H-BRCT, including the E3 ubiquitin ligase Tom1. Based on these findings, we propose a phospho-peptide consensus motif for interaction with Sir4 H-BRCT and Dbf4 H-BRCT. Ablation of the Sir4 H-BRCT phospho-peptide interaction disrupts SIR-mediated repression and perinuclear localization. In conclusion, the Sir4 H-BRCT domain serves as a hub for recruitment of phosphorylated target proteins to heterochromatin to properly regulate silencing and nuclear order.

Introduction

Chromatin-mediated silencing is critical for eukaryotic genome stability and cell survival. In budding yeast, *S. cerevisiae*, silent chromatin is maintained by the silent information regulator (SIR) complex which represses transcription in subtelomeric regions and the homothallic mating-type loci (Rine & Herskowitz, 1987; Aparicio *et al*, 1991; Hecht *et al*, 1995; Kueng *et al*, 2013). The SIR complex, composed of the NAD$^+$-dependent histone deacetylase Sir2, the histone binding protein Sir3, and the 152 kDa scaffold protein Sir4, serves as a model system to study epigenetically silenced heterochromatin in higher eukaryotes (reviewed in refs Gartenberg & Smith, 2016; Oppikofer *et al*, 2013). Repression of genes in *S. cerevisiae* is initiated by DNA-binding proteins repressor activator protein 1 (Rap1) (Shore *et al*, 1987; Moretti *et al*, 1994), ARS-binding factor 1 (Abf1) (Sussel & Shore, 1991), and origin recognition complex subunit 1 (Orc1) (Brand *et al*, 1987), which recognize specific DNA sequences located in silencer elements or telomeric repeats. Binding of these factors initiates chromatin repression by the recruitment of Sir1 and/or a hetero-trimeric SIR complex that can spread along the chromatin fiber by oligomerization (Rudner *et al*, 2005; Cubizolles *et al*, 2006; Swygert *et al*, 2018). Central to nucleation is Rap1, which binds with high affinity to silencer elements and telomeric DNA (Buchman *et al*, 1988); it then interacts with Sir4 to recruit Sir2 to nucleosomes, allowing Sir2 to deacetylate the N-terminal histone H4 tail (Moretti *et al*, 1994; Hecht *et al*, 1995; Imai *et al*, 2000; Tanner *et al*, 2000). Hypoacetylated histone H4, in particular the deacetylated H4K16 residue, serves as a high-affinity site for the Sir3 bromo-adjacent homology (BAH) domain, helping assemble the SIR2,3,4 complex on nucleosomes (Connelly *et al*, 2006; Onishi *et al*, 2007; Oppikofer *et al*, 2011). From such nucleation sites, successive histone deacetylation by Sir2, followed by Sir3 binding, and Sir3 homo- and hetero-dimerization with the Sir2-4 subcomplex, allows spreading of a repressed chromatin structure along the DNA fiber (Strahl-Bolsinger *et al*, 1997; Rusche *et al*, 2002; Kueng *et al*, 2013).

Sir proteins have been studied extensively over the last 25 years, and crystal structures of individual domains have been determined to understand function and structural organization of transcriptional silencing (reviewed in ref. Oppikofer *et al*, 2013). Sir4 binds both Sir2 and Sir3, and therefore is thought to provide a scaffold for SIR complex formation (Hecht *et al*, 1996; Moazed *et al*, 1997; Rudner

et al, 2005). Sir4 comprises several well-studied domains (Figs 1A and EV1). The Ku-binding motif (KBM, residues 104–115; green box in Fig 1A) of Sir4 is involved in recruiting telomerase to telomeres through interaction between telomere-bound Sir4 and the Ku-telomerase complex (Schober *et al*, 2009; Chen *et al*, 2018). yKu80/yKu70 interacts with Sir4 regions 1–270 and 747–1,358 to recruit the SIR complex to telomeres and facilitates anchoring of silenced chromatin at the nuclear periphery (Tsukamoto *et al*, 1997; Laroche *et al*, 1998; Hediger *et al*, 2002; Luo *et al*, 2002; Roy *et al*, 2004; Schober *et al*, 2009). Sir4 is also required for Ku-mediated telomere lengthening and telomerase recruitment by interaction with the yKu70/80 heterodimer (Ferreira *et al*, 2011; Hass & Zappulla, 2015). The Sir2-interacting domain (SID, residues 737–893; blue box in Fig 1A) is important for SIR complex assembly and allosteric Sir2 deacetylase regulation (Hsu *et al*, 2013). The SID also provides a link to the nuclear periphery through interaction with the N-terminus of Mps3 (Bupp *et al*, 2007; Schober *et al*, 2009).

A more central Sir4 domain called partitioning and anchoring domain (PAD, residues 950–1,262; white box in Fig 1A) mediates binding of silenced heterochromatin to the nuclear envelope by binding a C-terminal fragment of Esc1 (establishes silent chromatin 1) (Ansari & Gartenberg, 1997; Andrulis *et al*, 2002; Taddei *et al*, 2004). The Sir4 PAD also interacts with the integrase of the Ty5 retrotransposon to integrate Ty5 into heterochromatic regions at telomeres and silent mating-type loci. Both, Esc1 and Ty5 seem to use a short proline-rich motif to bind the Sir4 PAD. Furthermore, Ty5 binding to Sir4 is mediated by post-translational modifications, such as phosphorylation (Zou *et al*, 1996; Dai *et al*, 2007; Brady *et al*, 2008). Finally, an N-terminal region of the Ubp10 deubiquitinase (residues 109–133) has been shown to interact with the Sir4 PAD to remove H2B ubiquitin marks, a prerequisite for heterochromatin formation (Reed *et al*, 2015; Zukowski *et al*, 2018).

Despite this rich array of interactors, little is known regarding the structural basis of Sir4 PAD function. In contrast, the C-terminal coiled-coil domain (CC, residues 1,271–1,347; magenta box in Fig 1A) of Sir4 is structurally well characterized. It drives Sir4 dimerization, which is thought to enable interactions with Sir3 and yKu70 (Chang *et al*, 2003; Murphy *et al*, 2003). The Rap1 interaction sites have been mapped to Sir4 regions 142–591 and 839–1,358, while Sir3 binding seems to require Sir4 residues 745–1,172 (Moretti *et al*, 1994; Luo *et al*, 2002). Interestingly, the C-terminal half of Sir4 is sufficient for repression at *HM* loci, whereas repression at telomeres requires the Sir4 N-terminus as well (Kueng *et al*, 2012). In conclusion, Sir4 can be described as a multi-domain protein that serves as the central scaffolding subunit of yeast heterochromatin.

BRCT domains (named after the BRCA1 C-terminal domain) are signaling modules that occur as single or tandem units in numerous proteins, especially those involved in DNA damage response (Leung & Glover, 2011). These 90–100 amino acid residue-long domains typically mediate protein–protein interactions. With the exception of the Dbf4 BRCT domain, all BRCT domains studied to date comprise a four-stranded parallel β-sheet surrounded by three α-helices: β1α1β2β3α2β4α3. The Dbf4 BRCT domain, however, requires an additional N-terminal α-helix that stabilizes the BRCT core and is therefore named helix-BRCT (H-BRCT) (Matthews *et al*, 2012). The Dbf4 H-BRCT occurs as a single unit and is thought to be deficient for phospho-peptide interaction.

Here, we identify a second example of the non-canonical H-BRCT domain in the Sir4 protein. We show that the Sir4 H-BRCT domain serves as the functional module of the Sir4 PAD, and we describe its interaction with phosphorylated targets. By X-ray crystallography, we establish the structural basis of Esc1-mediated recruitment of telomeres to the nuclear periphery by the Sir4 H-BRCT domain and describe how short phosphorylated sequence stretches in Ty5 and Ubp10 are recognized in a structurally similar fashion. Structure-guided point mutations in the Sir4 H-BRCT domain that disrupt its ability to bind cognate phospho-peptides phenotypically mimic *sir4Δ* and result in Sir4 mislocalization from perinuclear foci and an inability to silence transcription. Furthermore, we reveal a previously uncharacterized phospho-dependent interaction of the Sir4 H-BRCT domain with the Tom1 E3 ubiquitin ligase and the centromere-binding factor Cbf1. Based on these structural, biochemical, and cell biological findings, we hypothesize that the Sir4 H-BRCT domain provides a platform for proteins with a phosphorylated consensus motif [LIM]-x-[ST]-[SDE]-[PF]-[PLM], which in turn regulate heterochromatin silencing. We further demonstrate that the related Dbf4 H-BRCT also interacts selectively with phospho-peptides.

Results

Identification and structure determination of the Sir4 H-BRCT

In order to explore the mechanisms by which Sir4 carries out its various functions (Fig 1A), we performed a deep analysis of the Sir4 protein sequence by employing structural bioinformatics tools. We used HHPRED (Soding *et al*, 2005) to identify new putative domains in Sir4 by searching for remote homologs of known structure in the Protein Data Bank (PDB). To our surprise, there was a significant hit (probability 100%, *E*-value = 1.9e-32) spanning Sir4 region ∼ 960–1,080 (located in the first half of the PAD, orange box in Fig 1A) suggesting distant homology to the H-BRCT domain of the *S. cerevisiae* CDC7-DBF4 kinase regulatory subunit Dbf4 (sequence identity 18%). The Dbf4 H-BRCT domain has been shown to interact with the forkhead-associated domain 1 (FHA1) of the Rad53 kinase in a phosphorylation-independent manner (Matthews *et al*, 2012, 2014; PDB ID 3QBZ). Protein disorder prediction on the GeneSilico MetaDisorder server (Kozlowski & Bujnicki, 2012) confirmed the Sir4 sequence stretch ∼ 950–1,100 to be structured. Multiple sequence alignments between several yeast Sir4 sequences and the H-BRCT domain of budding yeast Dbf4 were used (Fig 1B) for designing a Sir4 expression construct encompassing the putative domain. DNA coding for Sir4 residues 961–1,085 was cloned into an expression vector adding a N-terminal hexa-histidine tag, and the protein was expressed in *Escherichia coli* followed by purification to homogeneity. The protein crystallized in space group *C*2 with one molecule per asymmetric unit and the structure was determined by the single anomalous diffraction (SAD) method using a selenomethionine labeled derivative of the protein. The final structure was then obtained by refining the initial model against diffraction data collected from a native protein crystal at a resolution of 1.1 Å resulting in a model with good R-factors ($R = 11.7\%$, $R_{\text{free}} = 14.2\%$) and stereochemical parameters (Table EV1).

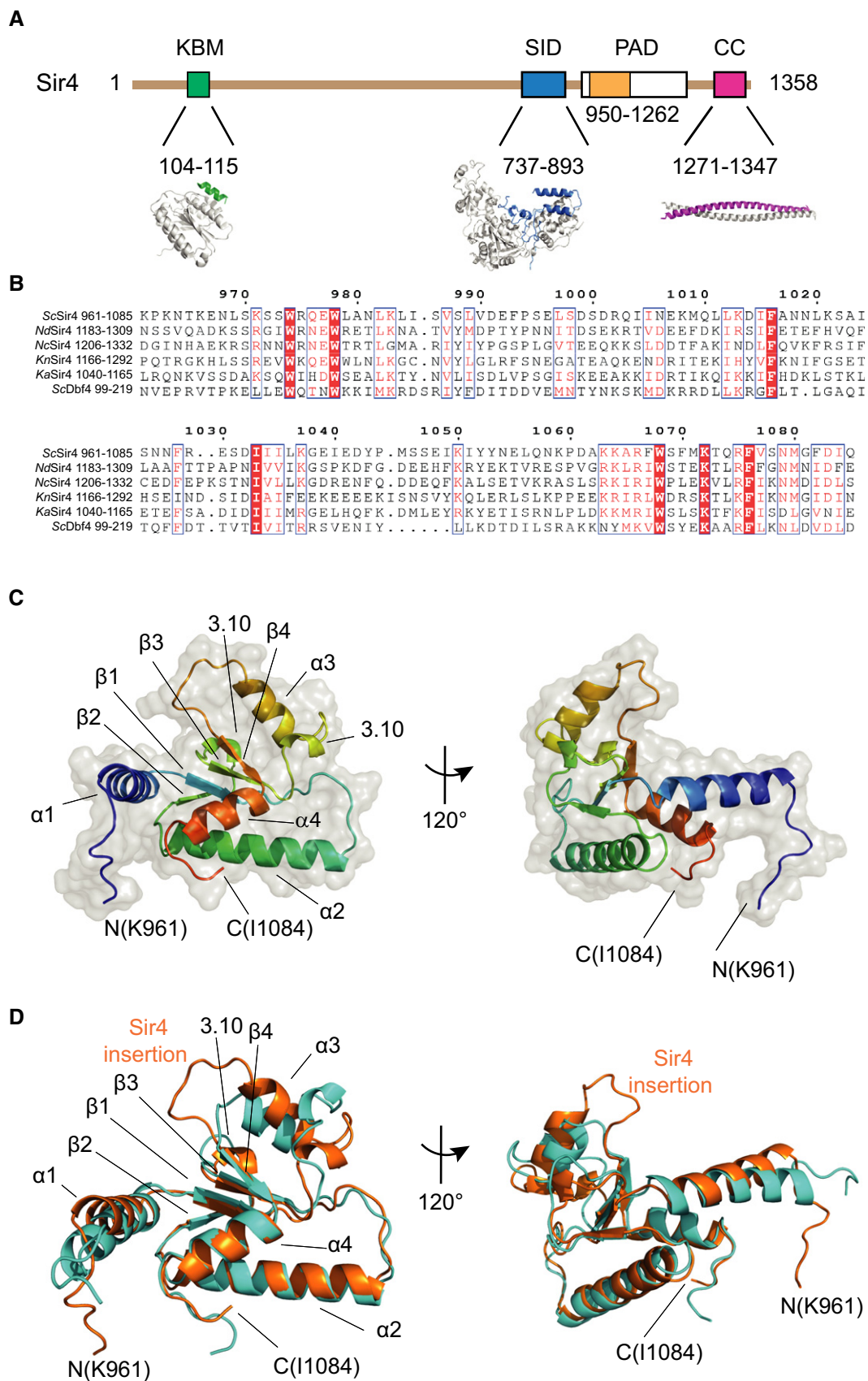Figure 1.

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Figure 1. Crystal structure of Sir4 H-BRCT.

A   Domain architecture of Sir4. Crystal structures have been determined for the Sir4 Ku80-binding motif (KBM) in complex with Ku80, the Sir4-interacting domain (SID) in complex with Sir2, and the Sir4 self-dimerization coiled-coil module (CC) (PDB 5Y59, 4IAO, and 1NYH, respectively). Sir4 H-BRCT (orange box, this work) locates to the first half of the partitioning and anchoring domain (PAD) which recruits silenced chromatin to the nuclear envelope.

B   ClustalO (Sievers *et al*, 2011) multiple sequence alignment of various yeast Sir4 proteins focusing on the putative new structural domain and comparison with *Saccharomyces cerevisiae* Dbf4 H-BRCT. Sir4 sequences: ScSir4 (*S. cerevisiae*, UniProt P11978), NdSir4 (*Naumovozyma dairenensis*, G0W8P4), NcSir4 (*Naumovozyma castellii*, G0VBN1), KnSir4 (*Kazachstania naganishii*, J7S2D5), and KaSir4 (*Kazachstania africana*, H2AVL7).

C   Scheme of the Sir4 H-BRCT crystal structure in two orientations rotated by ~ 120° around a vertical axis. Colors gradually vary from blue (N-terminus) to red (C-terminus), while the surface is shown in gray (transparent). Termini and secondary structural elements are labeled.

D   Superposition of Sir4 H-BRCT (orange) onto the H-BRCT domain of Dbf4 (PDB 3QBZ, cyan) in two orientations as in (C). Secondary structural elements, termini, and the elongated Sir4 loop connecting helix α3 and strand β4 are labeled.

Overall structure of Sir4$^{(961–1,085)}$

The structure of Sir4$^{(961–1,085)}$ displays clear electron density at high resolution and folds into a BRCT-like fold with a four-stranded parallel β-sheet in the center surrounded by four α-helices and two short stretches of 3.10-helices (Fig 1C). The structure superimposes well onto the structure of the H-BRCT domain of Dbf4 (PDB 3QBZ) with a r.m.s.d. of only 1.4 Å over 433 atoms (Fig 1D). Similar to the H-BRCT domain of Dbf4, Sir4$^{(961–1,085)}$ also possesses an additional α1 helix at the N-terminus and has an elongated α2 helix, thereby featuring the two unique structural elements which are different from canonical BRCT domain structures. Hence, we will refer to the newly discovered Sir4 domain from here onwards as H-BRCT. Besides the overall structural similarity of protein backbones, H-BRCT domains of Sir4 and Dbf4 differ in the conformation of loops between β-strands β2 and β3, between strand β3 and helix α3, in the length of the loop between helix α3 and strand β4 where a seven-residue insertion is found in Sir4, and in the position and angles of helices α1 and α3 (Fig 1D).

Sir4 H-BRCT domain does not interact with Rad53 FHA1

H-BRCT domains of Sir4 and Dbf4 are structurally very similar but Dbf4 residues critical for interaction with the FHA1 domain of Rad53 (black and blue asterisks in Fig 2A) are only partially conserved in Sir4. Nevertheless, we wondered whether Sir4 H-BRCT would also regulate Rad53 activity by binding to the FHA1 domain in a similar way. Recently, the crystal structure of Dbf4 H-BRCT in complex with the FHA1 domain of Rad53 bound to a CDC7 peptide was determined using an engineered single chain chimera of the two proteins revealing a bipartite interaction surface (Almawi *et al*, 2016). Superposition of Sir4 H-BRCT onto the Dbf4 H-BRCT-Rad53-CDC7 complex (Fig 2B) identified Dbf4 H-BRCT helix α1 residues contributing to interface I (black dashed circle in Fig 2B) as partially conserved in Sir4, while interface II (blue dashed circle in Fig 2B), mainly formed by the loop connecting Dbf4 H-BRCT helix α3 and strand β4, was not conserved as a seven-residue insert replaces it (orange bar in Fig 2A). This comparatively longer Sir4 H-BRCT loop is quite flexible in the *C*2 crystal structure (Fig 2B) which accounts for the partial disorder of residues Asp1061 and Ala1062. The Sir4 H-BRCT loop lacks an aromatic residue corresponding to Tyr198 (Dbf4 H-BRCT, blue arrow in Fig 2A) which contributes most of the binding energy for the FHA1 domain (computed using PISA, https://www.ebi.ac.uk/pdbe/pisa/) in this region. The lysine residue of Sir4 H-BRCT at this position (Lys1064, orange arrow in

Fig 2A) cannot provide the hydrophobic surface necessary for binding and would prevent, along with the increased steric requirements of the longer Sir4 H-BRCT loop, any potential Rad53 FHA1 interaction. To test whether Sir4 H-BRCT interacts with Rad53 FHA1, we performed *in vitro* pull-down experiments. His-tagged Rad53 FHA1 immobilized on Ni-NTA magnetic beads failed to interact with untagged Sir4 H-BRCT, but interacted with Dbf4 H-BRCT (Fig EV2A). In addition, Sir4 H-BRCT and Rad53 FHA1 did not co-elute as a heterodimer as analyzed by size exclusion chromatography coupled with multi-angle light scattering (SEC-MALS; Fig EV2B and C). Therefore, we conclude that the H-BRCT domains of Sir4 and Dbf4 may bind different interactors.

Structural basis of Sir4 H-BRCT interaction with Esc1, Ty5, and Ubp10 phospho-peptides

Analysis of the electrostatic surface potential of Sir4 H-BRCT displayed a clearly defined basic patch formed by conserved residues Arg1066, Lys1072, and Arg1075, which was located in a pocket shaped by conserved helix α1 residues Trp974 and Trp978, the elongated Sir4 H-BRCT loop 1,058–1,065 and the core of the H-BRCT domain (Fig 3A). Given that the Sir4 H-BRCT is found on the PAD, which has been shown to interact with several other proteins (Figs 1A and EV1), we hypothesized that the Sir4 H-BRCT domain is responsible for PAD interactions. Interestingly, the Ty5 motif that interacts with the first part of the Sir4 PAD has been shown to be dependent on a single phosphorylation site and a related proline-rich sequence identified in Esc1 also lost binding to Sir4 upon mutation of a serine to alanine (Dai *et al*, 2007; Brady *et al*, 2008). Based on these findings, we hypothesized that Sir4 H-BRCT recognizes a phospho-epitope on Esc1. To test this hypothesis, we used two phosphorylated Esc1 peptides for co-crystallization experiments with Sir4 H-BRCT encompassing a conserved proline-rich motif (IPSTDLP**pS**DPPSDKEE and IPSTDLPSDPP**pS**DKEE, residues 1,443–1,458) which is part of the minimal sequence stretch (residues 1,440–1,473) mapped for interaction with Sir4 (Andrulis *et al*, 2002). These Esc1 phosphorylation sites (pS1450 and pS1454) were experimentally determined in a global analysis of cyclin-dependent kinase 1 substrates involved in cell cycle control in yeast (Holt *et al*, 2009). In addition, we performed co-crystallization experiments with Sir4 H-BRCT using the well-characterized Ty5 motif needed for Ty5 retrotransposon integration at heterochromatic loci in its Ser1095 phosphorylated state (pS1095, ESPPSLDS**pS**PPNTSFNA, residues 1,087–1,103; Dai *et al*, 2007; Brady *et al*, 2008), and a proline-rich peptide that we identified in the Ubp10 sequence stretch
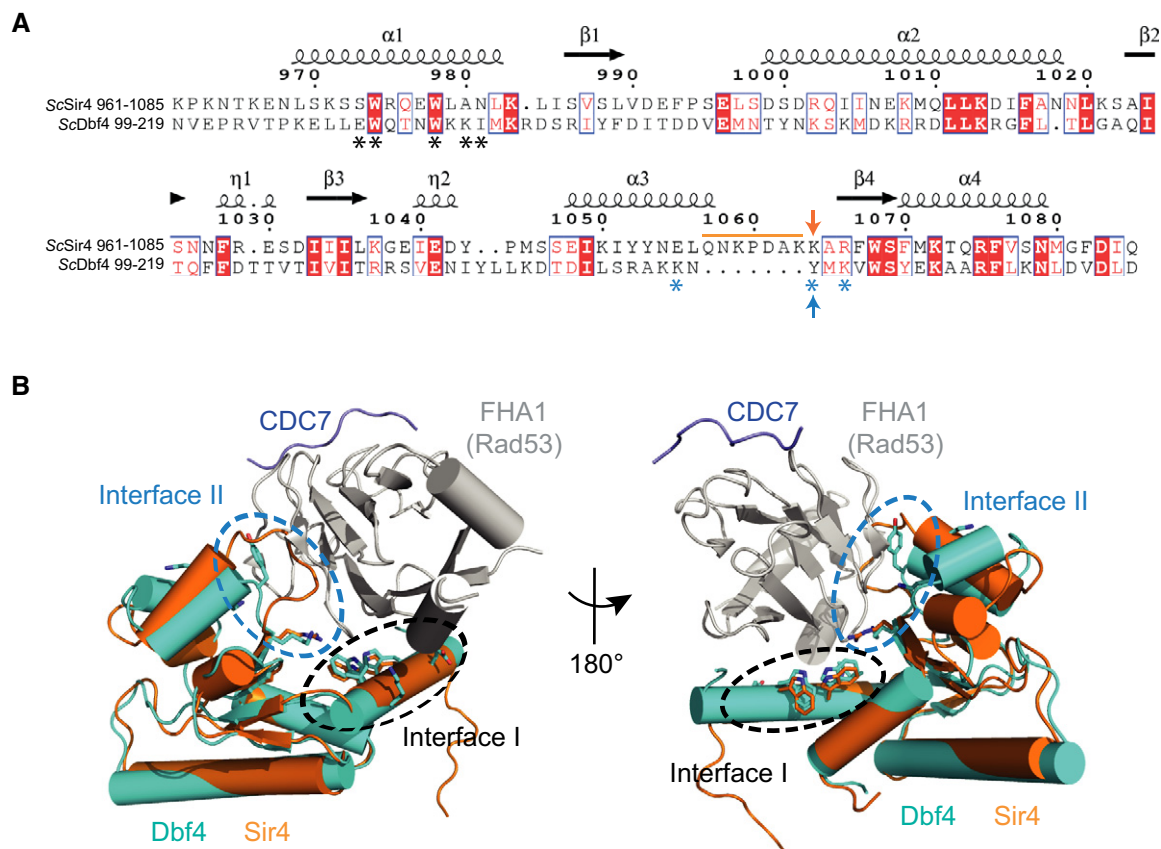
Figure 2. Sir4 H-BRCT structural comparison with Dbf4 H-BRCT and Rad53 FHA1 binding.

A Structure-based sequence alignment between H-BRCT domains of Sir4 (top) and Dbf4 (PDB 3QBZ, bottom). Secondary structural elements present in the Sir4 H-BRCT crystal structure are displayed above the Sir4 sequence (α, α-helix; β, β-strand; η, 3.10 helix). Dbf4 residues strongly contributing to Rad53 FHA1 binding are highlighted with black (interface I) and blue (interface II) asterisks. The elongated loop connecting helix α3 and strand β4 in Sir4 H-BRCT is labeled with an orange bar, and the Tyr198 (Dbf4) to Lys1064 (Sir4) exchange is highlighted with colored arrows.

B Superposition of Sir4 H-BRCT (orange) onto the Dbf4-Rad53-CDC7 complex (cyan, gray, blue) (PDB 5T2S) in two orientations rotated by ~ 180° around a vertical axis. Helices are displayed as barrels, and protein–protein interfaces between Dbf4 and FHA1 of Rad53 are highlighted with dashed circles in black (interface I) and blue (interface II).

(residues 109–133) that had been recently shown to interact with Sir4 (Zukowski *et al*, 2018). Based on sequence comparisons with Esc1 and Ty5, we hypothesized that the Ubp10-Sir4 interaction might also be phosphorylation dependent and therefore used the Ubp10 peptide (LSTELS**pT**EPPSS, residues 117–128) in its Thr123 phosphorylated state (pT123).

Crystals were obtained, and structures of Sir4 H-BRCT in complex with Esc1^{pS1450}, Ty5^{pS1095}, and Ubp10^{pT123} peptides were determined by the molecular replacement method at 2.5, 3.0, and 2.18 Å resolution, respectively (Table EV1). All three peptides interact with Sir4 H-BRCT using the positively charged patch created by Arg1066, Lys1072, and Arg1075 which is surrounded by helix α1/α4 and β-strand β4, and feature a highly similar conformation (Figs 3B and EV3). We did not obtain crystals of Sir4 H-BRCT in complex with Esc1^{pS1454} peptide and therefore only focused on the Esc1^{pS1450} peptide in subsequent experiments. The Esc1 pSer1450 phospho-group is tightly anchored through electrostatic interactions with Arg1066, Trp1068, and Lys1072 side chains. It allows the peptide to form a sharp turn to use the aromatic indole rings of Sir4 H-BRCT

Trp974 and Trp978 for hydrophobic interaction with Esc1 residues Leu1448 and Pro1452/1453. This way, the Pro1449 carbonyl group is in ideal hydrogen bonding distance (2.9 Å) to NE1 atoms of the same Trp974/978 residues, while the Leu1448 carbonyl can interact with the Arg1066 guanidinium group (Fig 3C). In the case of Ty5, the pS1095 phospho-group is shifted by one residue toward the double proline motif and is therefore bound by Sir4 H-BRCT residues Lys1072 and Arg1075, while the Ser1094 hydroxyl group directly contacts Arg1066 (which is also in contact with a sulfate ion from the crystallization buffer; Fig 3D and EV3B). Other contacts are similar as in the case of Esc1, with Ty5 Leu1092 and Pro1096/1097 making hydrophobic contacts with Sir4 H-BRCT Trp974/978, and Ty5 Asp1093 hydrogen bonding the same residues using its carbonyl group. The Ubp10^{pT123} peptide binds in a similar way by positioning the phospho-group (this time located on a threonine) in the same place as the Esc1 peptide, where it interacts with Arg1066, Trp1068, and Lys1072 (Fig 3E). The overall binding mode is again conserved with Ubp10, where Leu121 and Pro125/126 make hydrophobic contacts with Sir4 H-BRCT Trp974/978 and Ubp10

Ser122 mediates hydrogen bonding to the same residues using its carbonyl group. In addition, the Ubp10 Glu124 side chain is long enough to hydrogen bond to Sir4 Arg1075, while the Ser118 backbone carbonyl makes an additional weak hydrogen bond to the Arg1066 side chain of Sir4 H-BRCT, extending the Sir4-binding sequence motif toward the N-terminus of the peptide.

Having characterized the binding mode of Sir4 H-BRCT to Esc1, Ty5, and Ubp10 phospho-peptides at atomic resolution, we used microscale thermophoresis (MST) to determine the affinity of Sir4 H-BRCT for these peptides in phosphorylated and non-phosphorylated states (Fig 3F, and Tables EV2 and EV3). All three peptides bound Sir4 H-BRCT domain in a phosphorylation-dependent
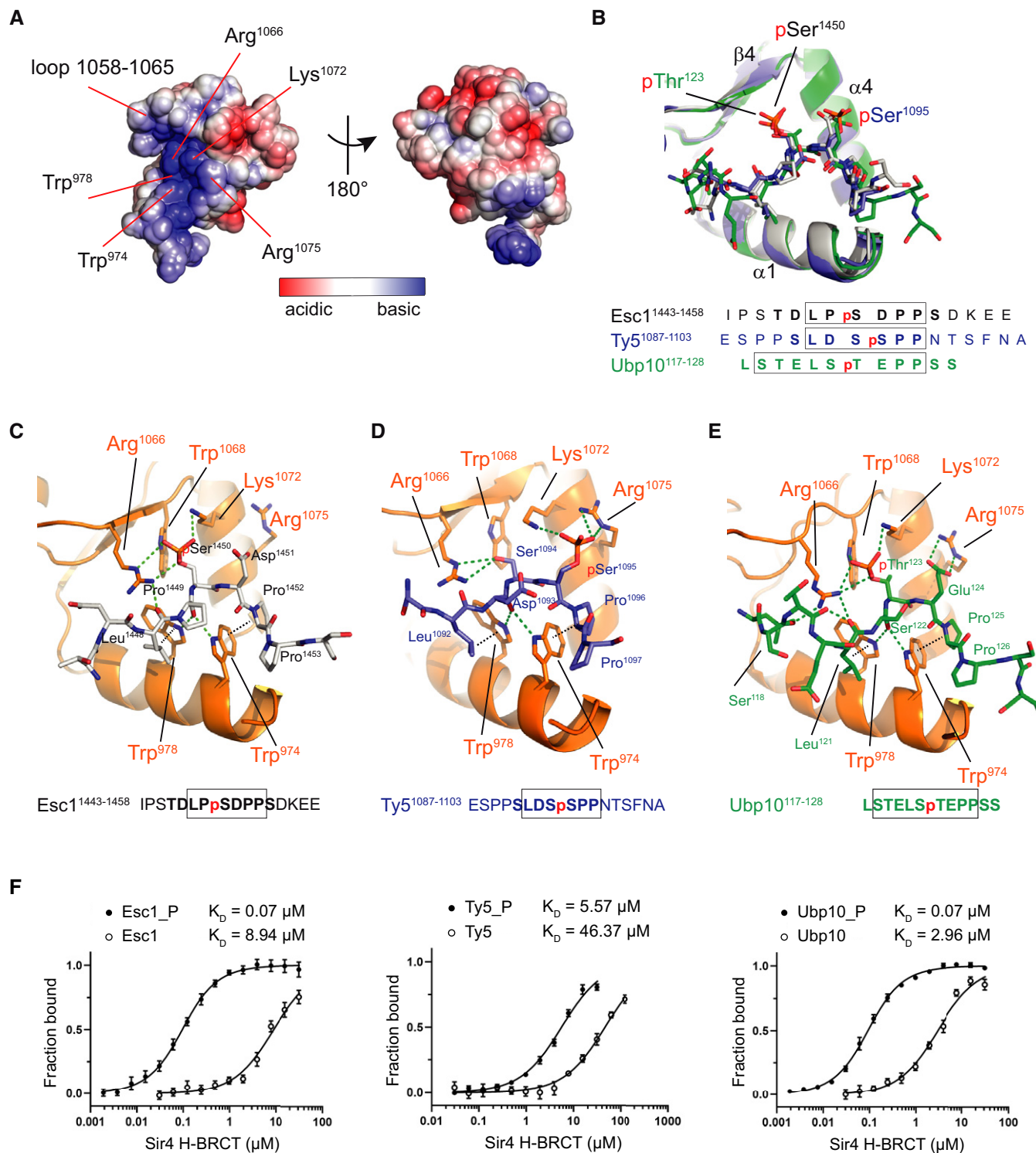

Figure 3.

manner with an equilibrium dissociation constant, K_D, in the nanomolar range for Esc1 and Ubp10 (0.07 μM) and low micromolar range for Ty5 (5.57 μM).

## Sir4 RKR residues are critical for Sir4 localization and telomere clustering at the nuclear periphery

In budding yeast, heterochromatin is anchored to the nuclear periphery through the inner membrane-associated proteins Esc1 (Andrulis *et al*, 2002; Hediger *et al*, 2002; Taddei *et al*, 2004) and Mps3 (Bupp *et al*, 2007; Schober *et al*, 2009). Cells lacking Esc1 fail to tether heterochromatin to the nuclear envelope and are characterized by dispersed telomeres and telomere-associated SIR foci (Hediger *et al*, 2002; Gartenberg *et al*, 2004; Taddei *et al*, 2004). Our structural and biochemical insights highlighted the importance of the Sir4 H-BRCT positively charged triad (Arg1066, Lys1072 and Arg1075) in binding Esc1, Ty5, and Ubp10 phospho-peptides. To test the functional significance of phospho-peptide binding, we mutated these three residues to alanines in Sir4 (hereafter named *sir4* RKR), thereby disrupting the phospho-peptide interaction, and tested telomere recruitment in the *sir4* RKR mutant. We tagged Nup49, an integral nuclear pore protein as a marker of the nuclear envelope (NE), with red fluorescent protein (RFP), and either Sir4 wild-type (wt) or the *sir4* RKR mutant with green fluorescent protein (GFP) and expressed fusion proteins under their endogenous promoters. Yeast strains used are summarized in Table EV4. We then analyzed localization of Sir4-GFP and Nup49-RFP in both wt and RKR strains by fluorescence microscopy (Fig 4A–C). As expected in wt cells, Sir4 clustered in 4–6 foci per nucleus representing the Esc1-mediated telomere assembly at the NE. However, Sir4 protein levels were reduced in the RKR mutant as seen by a reduced number of Sir4 foci (1–3) per nucleus and a ∼ 30% decrease in the total nuclear intensity of the Sir4-GFP signal (Fig 4B, dark gray circles). Since Sir4 protein levels greatly impact Sir4 function (Cockell *et al*, 1995; Smith *et al*, 1998), we performed competition experiments to study the role of Sir4 RKR mutations without affecting Sir4 protein levels. In our competition assay, we overexpressed the Sir4 H-BRCT domain in wild-type yeast, to allow the overexpressed Sir4 H-BRCT fragment to compete with endogenous Sir4 for binding to Sir4's phosphorylated interactors. Importantly, overexpression of the Sir4 H-BRCT domain did not reduce endogenous Sir4 protein levels (Fig 4B, orange circles), but caused a ∼ 40% reduction in Sir4-containing telomere clusters at the NE (Fig 4C, orange bar). Overexpression of the mutant Sir4 RKR H-BRCT showed no Sir4 focus mislocalization (Fig 4C, light blue bar). Notably, protein levels of overexpressed Sir4 wt and mutant H-BRCT domains were similar and both domains showed similar biochemical characteristics when purified to homogeneity (Fig EV4). Given that Sir4 coincides with telomeric foci, our observations argue that mutations that ablate the binding of Sir4 H-BRCT to phospho-proteins alter both Sir4 localization and telomere recruitment to the nuclear rim.

Sir4 H-BRCT RKR residues are critical for heterochromatin silencing

To test the role of the RKR motif in heterochromatin silencing, we performed Sir4 H-BRCT competition experiments using previously established silencing assays (Marshall *et al*, 1987; Kueng *et al*, 2012). Yeast strains used in this study are summarized in Table EV4. To monitor silencing at telomeres, we scored the expression of a *URA3* reporter gene inserted at telomere 7L using a colony growth assay on media lacking uracil (Fig 4D). We used a strain that lacks Ppr1, a transcription factor that induces high-level *URA3* expression in the absence of uracil (Renauld *et al*, 1993). As expected, in cells transformed with an empty plasmid, endogenous Sir4 was able to silence the *URA3* reporter gene resulting in lack of growth on uracil-deficient media (Fig 4D, row 1). Overexpression of wt Sir4 H-BRCT was able to disrupt repression of the reporter gene to allow colony growth (Fig 4D, row 2), phenocopying a strain lacking *sir4* (Fig 4D, row 4). On the other hand, overexpression of the mutant Sir4 RKR H-BRCT showed no *URA3* silencing defect (Fig 4D, row 3). Therefore, competing out phospho-interactors of the Sir4 H-BRCT domain disrupted SIR-mediated silencing in subtelomeric domains.

We next tested whether the Sir4 RKR motif is essential for silencing a reporter gene in another chromosomal context. For this purpose, we used a *HMR-EΔB::TRP1* reporter strain that lacks the B sequence (Abf1-binding site) within the E silencer (Brand *et al*, 1987) at the endogenous *HMR* locus (Fig 4E). Similar to the subtelomeric silencing defect, overexpression of wt Sir4 H-BRCT, but not Sir4 RKR H-BRCT, disrupted repression of the *TRP1* reporter
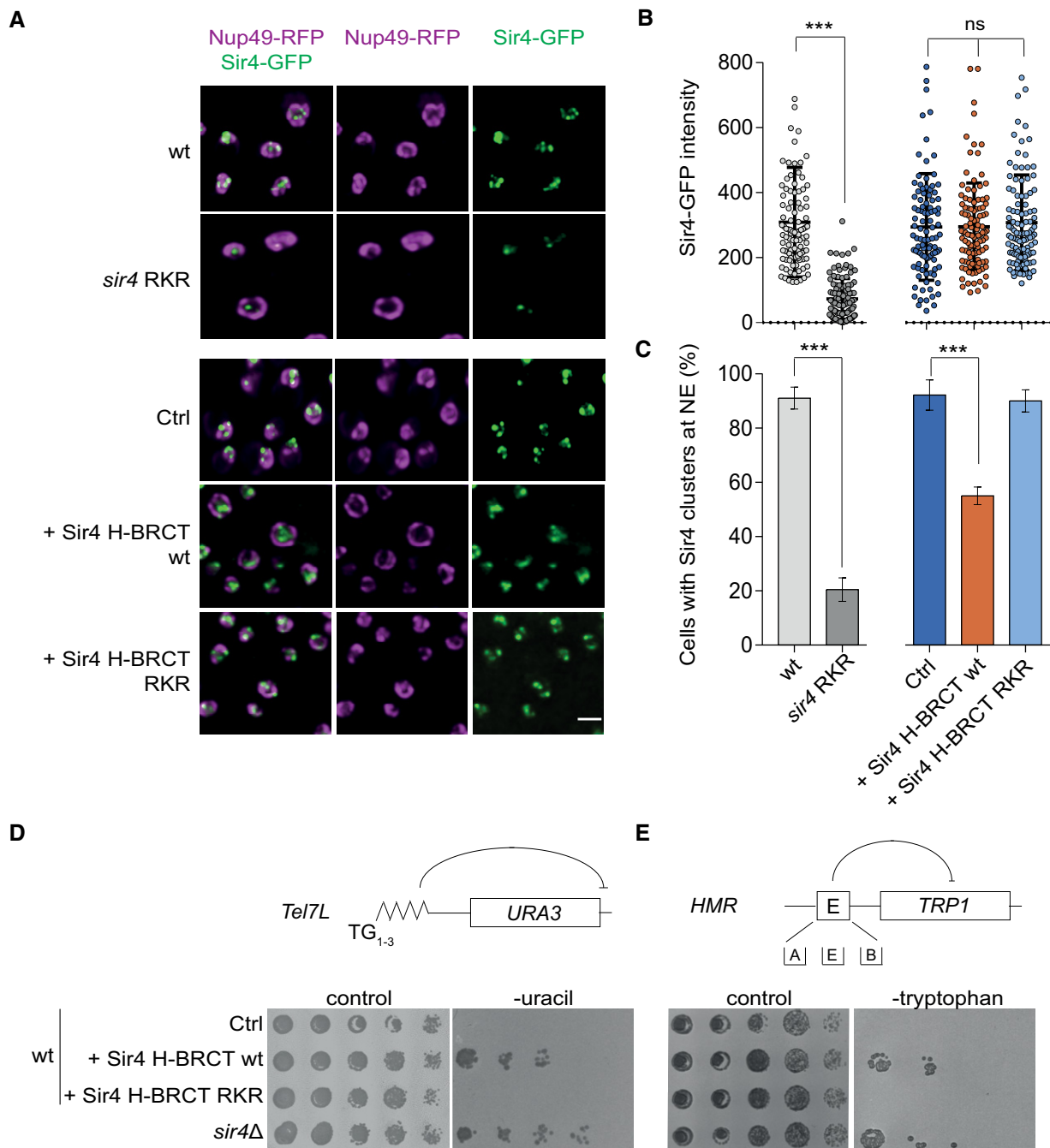
Figure 4. Sir4 RKR residues are important for heterochromatin silencing and proper localization of Sir4 at the nuclear periphery.

A Example images of Nup49-RFP Sir4-GFP in wild-type (wt) cells (GA-10372; row 1); *sir4* RKR cells (GA-10373; row 2); wt cells transformed with either empty plasmid (row 3), plasmid overexpressing wt Sir4 H-BRCT (row 4), or plasmid overexpressing RKR mutant Sir4 H-BRCT (row 5). The bar indicates 2 μm.

B Quantification of mean fluorescence intensity of Sir4-GFP using > 100 cells. Error bars show the SD from three independent experiments.

C Quantification of cells containing Sir4 clusters at the nuclear envelope (NE). Data are represented as mean ± SD from three independent experiments where > 100 cells were counted.

D Top: Schematic representation of the TEL7L::URA3 reporter used to assay silencing at telomeres. Bottom: wt cells (GA-503) transformed with either an empty plasmid (row 1), plasmid overexpressing wt Sir4 H-BRCT (row 2), or plasmid overexpressing RKR mutant Sir4 H-BRCT (row 3). Cells lacking endogenous sir4 (GA-5822) (row 4). Cells were grown in the absence of uracil and plated as 1:5 serial dilutions onto control YPAD or YPAD lacking uracil plates and imaged after 2 days.

E Top: Schematic representation of the HMR-EΔB::TRP1 reporter gene locus used for assaying silencing at the HMR locus. Bottom: wt cells (GA-485) transformed with plasmids as in panel D. Cells lacking endogenous sir4 (GA-6888) (row 4). Cells were grown in the absence of tryptophan and plated as 1:5 serial dilutions onto YPAD or YPAD lacking tryptophan plates and imaged after 2 days.

Data information: (B, C) ***$P < 0.001$ (t-test).

gene at *HMR* and phenocopied a complete loss of *sir4*. In conclusion, these silencing assays suggest that the Sir4 RKR motif, which is essential for phospho-peptide interaction, is required both for heterochromatin silencing at telomeric and *HMR* loci, and for Sir4 focus formation at the NE.

Establishing the nuclear phospho-proteome of Sir4 interactors by mass spectrometry

Based on the versatility of the Sir4 H-BRCT domain in binding three different phospho-peptides (Esc1, Ty5, and Ubp10), we hypothesized that the H-BRCT domain might act as a general platform to recruit a variety of phosphorylated proteins to heterochromatin. To examine putative novel phospho-dependent interactors of the Sir4 H-BRCT domain globally, we performed immunoprecipitation followed by mass spectrometric analysis (IP-MS). The C-terminally Myc-tagged Sir4 H-BRCT domain, either wt or harboring phospho-peptide binding-deficient R1066A, K1072A, R1075A mutations, was expressed in *trans* from a plasmid in a *sir4Δ* background strain to specifically enrich for RKR-dependent interactors of the H-BRCT domain. Interacting proteins were immunoprecipitated from yeast cell extracts using ChromoTek Myc-Trap_MA beads in the presence of phosphatase inhibitors, and the purified material was reduced, alkylated, and digested for subsequent analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS; Fig 5A). Results are visualized in a volcano plot highlighting nuclear proteins enriched in Sir4 wt but not in Sir4 RKR mutant cells implying specific, phosphorylation-dependent interaction of identified proteins with the Sir4 H-BRCT domain (upper right quadrant; Fig 5B).

As expected, we found a strong enrichment of Esc1 in the wt, but not the mutant, Sir4 H-BRCT interactome. This validates our experimental approach and argues that Esc1 binds the Sir4 H-BRCT domain in a phosphorylation- and RKR motif-dependent manner. In addition, a set of RNA processing proteins previously not known to interact with Sir4 was identified. The U6 snRNA-associated Sm-like protein LSM6 and the DNA-directed RNA polymerase II subunit RPB1 (RPO21) were moderately enriched (1.68- and 1.66-fold compared to Esc1), while the mRNA-capping enzyme subunit beta protein CET1 and the ribosome biogenesis protein TSR1 were enriched just above threshold level. Interestingly, the E3 ubiquitin ligase Tom1 and the centromere-binding factor Cbf1 were highly enriched (3.61- and 3.78-fold, respectively, compared to Esc1) and are therefore likely to be novel Sir4 interactors.

To identify candidate phospho-peptides in Tom1 and Cbf1 that may be involved in Sir4 H-BRCT binding, we compared the observed phosphorylation sequences from our mass spectrometry analyses with consensus interaction motifs in Esc1, Ty5, and Ubp10. These putative interaction sites in Tom1 and Cbf1 were then tested for Sir4 H-BRCT binding using MST. Cy5 labeled Tom1 peptide 2,370–2,381 (HSREID**pS**FLEA) and Cbf1 peptide 149–160 (SLEGMT**pS**SPMES) bound Sir4 H-BRCT with affinities of $K_D = 35.06$ μM, and 15.96 μM, respectively. Since the measured affinities between Sir4 H-BRCT and Tom1/Cbf1 phospho-peptides were relatively weak, it is possible that there is an additional interface between Sir4 and Tom1/Cbf1 proteins. Nevertheless, the Sir4 binding was dependent on the presence of the phospho-group on Tom1 and Cbf1 peptides (Fig 5C, and Tables EV2 and EV3).

Because ubiquitination dynamics and protein turnover have been implicated in telomeric silencing (Burgess *et al*, 2012), we examined further if endogenous Sir4 stably binds Tom1 by performing co-immunoprecipitations. For this, we constructed HA-tagged Tom1 and Myc-tagged Sir4 in either wt or RKR backgrounds. Interacting factors were immunoprecipitated from whole cell extracts using Myc-Trap magnetic beads (ChromoTek), and the co-precipitation of Tom1-HA was monitored with an anti-HA antibody (Fig 5D). Indeed, Tom1-HA co-immunoprecipitated with wt Sir4-Myc (lane 1) but failed to co-immunoprecipitate with the mutant Sir4-RKR-Myc (lane 2). To confirm our hypothesis that the Tom1-Sir4 interaction is phosphorylation dependent, we treated our IP samples with lambda phosphatase to remove phosphorylation. In agreement with our IP-MS and MST results, the Sir4-Tom1 interaction was lost when cell extracts were treated with phosphatase, providing strong evidence that the Sir4-Tom1 interaction is phosphorylation-dependent. In conclusion, we identify Tom1 as a novel interactor of the Sir4 PAD domain and provide evidence that this interaction occurs through the Sir4 H-BRCT domain in a phosphorylation-dependent manner.

Dbf4 H-BRCT is also a phospho-peptide-binding module

With the exception of the elongated Sir4 H-BRCT loop encompassing residues 1,058–1,065, which includes the tyrosine (Dbf4, Tyr198) to lysine (Sir4, Lys1064) switch (Fig 2), there is high overall structural conservation between the Dbf4 H-BRCT and Sir4 H-BRCT domains. We therefore explored whether Dbf4 H-BRCT might also bind a range of phosphorylated target peptides like those that interact with Sir4 H-BRCT. The superposition of Dbf4 H-BRCT and Sir4 H-BRCT domains reveals that only Arg1066, the key phospho-anchoring residue in the Sir4 phospho-epitope-binding region involved in Esc1, Ubp10, Tom1, and Cbf1 interaction, is replaced by a lysine residue in Dbf4 H-BRCT (Lys200, black arrow in Fig 6A). This conservative change from an arginine to a lysine keeps the positive charge for electrostatic interaction with a phospho-group, which is a prerequisite to induce the sharp turn that brings the hydrophobic residues flanking the phospho-site in close contact with the double tryptophan motif (Trp974/978 in Sir4, Trp112/116 in Dbf4) located on the N-terminal α-helix of H-BRCTs. We tested whether Esc1^{pS1450}, Ty5^{pS1095}, Ubp10^{pT123}, Tom1^{pS2376}, and Cbf1^{pS155} peptides bind to purified Dbf4 H-BRCT using MST (Figs 6B and EV5, and Tables EV2 and EV3). We calculated equilibrium dissociation constants, K_D, of 0.9, 0.63, 75.46, and 172.43 μM for Esc1^{pS1450}, Ubp10^{pT123}, Tom1^{pS1095}, and Cbf1 ^{pS155} peptides, respectively, indicating a broad range from tight to weak binding. Binding was completely dependent on the phospho-group, given that non-modified peptides failed to interact. On the other hand, the Ty5 pS1095 peptide, in which the phospho-group is shifted toward the C-terminus by one residue, had no detectable affinity. Intriguingly, in our Sir4 H-BRCT-Ty5^{pS1095} co-crystal structure, we resolved a sulfate ion, which may serve as a Ty5 S1094 phosphorylation mimic, interacting with Sir4 R1066. Therefore, we performed MST binding assays in the presence of sulfate ions to mimic phosphorylation on S1094, to test whether pS1094/pS1095 is a more appropriate motif for Dbf4 H-BRCT binding to the Ty5 peptide. Indeed, we were able to measure weak binding ($K_D = 66$ μM) between Dbf4 H-BRCT and Ty5^{pS1095} peptide in the presence of sulfate ions (Fig EV6). Binding remained dependent

on the phosphorylation of S1095 since the peptide lacking phospho-modification showed no measurable binding, but these results suggest a preference for a double phosphorylation motif in Dbf4

H-BRCT interactions. Importantly, in comparison with Sir4 H-BRCT, we note that all ligands except Tom1 have roughly a tenfold lower K_D for their interaction with the Dbf4 H-BRCT domain. We assume
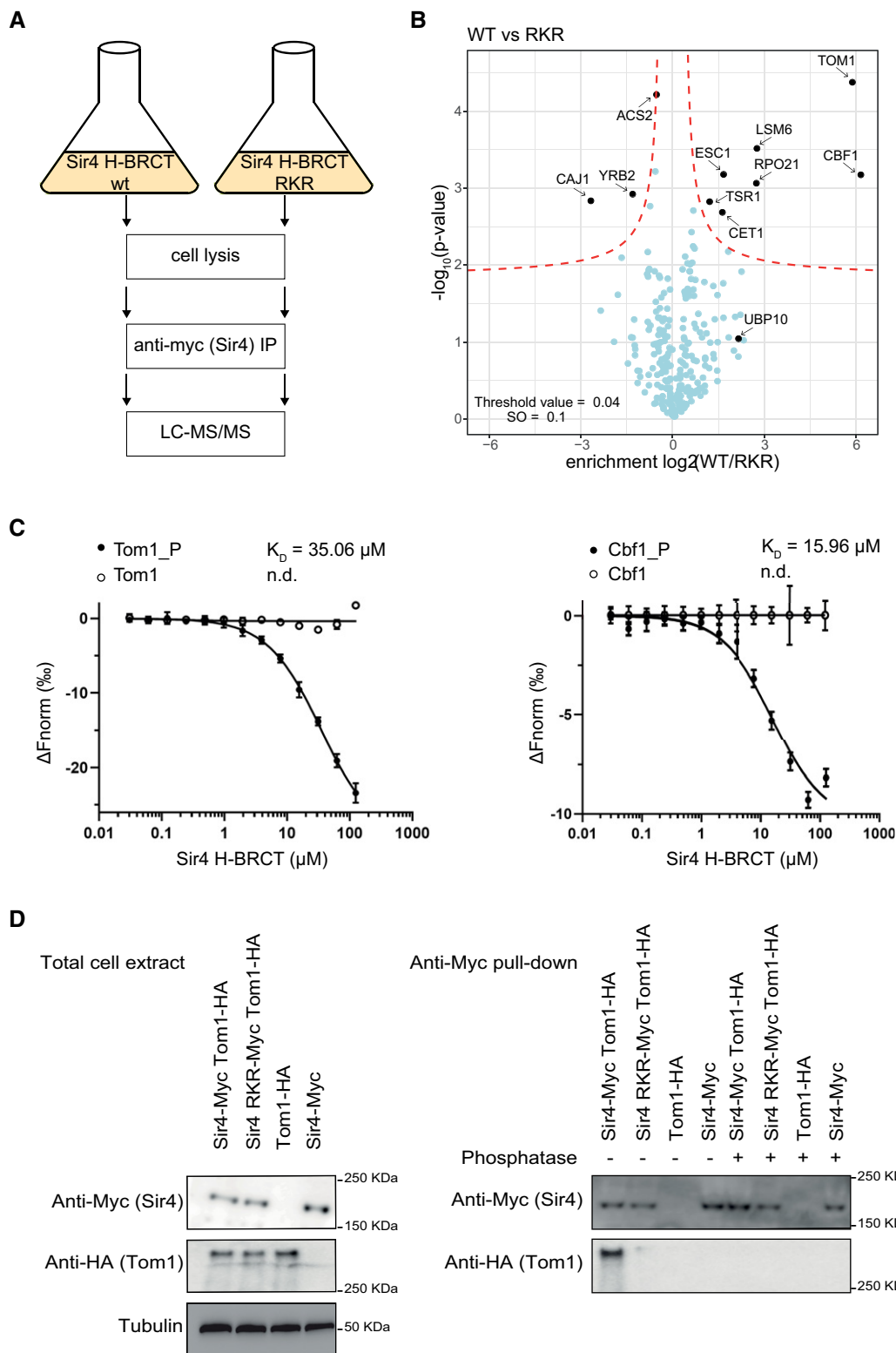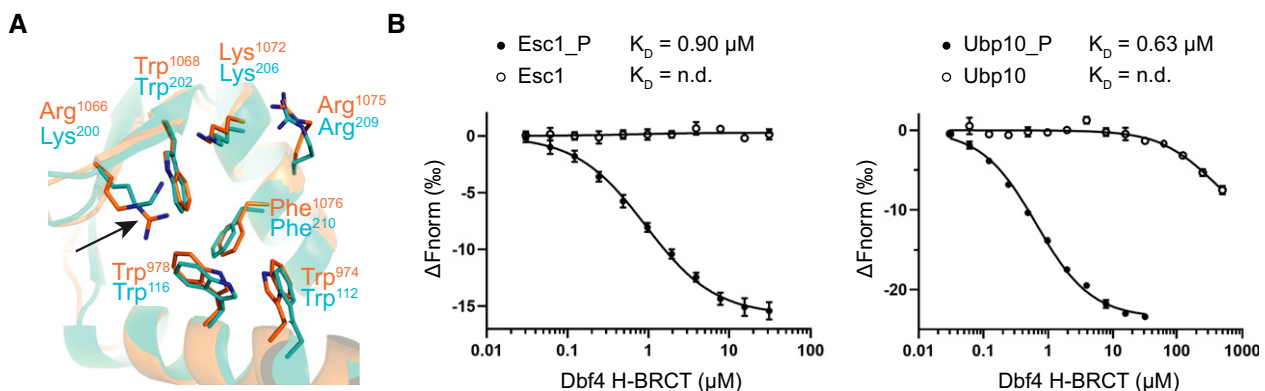

Figure 5.

that this is due to the replacement of Arg1066 in Sir4 by Lys200 in Dbf4. This underscores both the conservation and the fine-tuning possible within this important class of non-canonical BRCT domains. We conclude that the H-BRCT domains of Dbf4 and Sir4 both bind phospho-peptides, although with differences in selectivity and affinity.

Discussion

We describe a non-canonical H-BRCT domain in the budding yeast heterochromatin maintenance protein Sir4 and investigate in detail its capacity to interact with phosphorylated target proteins. BRCT domains are extensively studied phospho-peptide binder modules that were initially characterized in DNA damage response proteins, where they participate in a complex kinase signaling network that arrests the cell cycle in response to DNA damage and trigger DNA repair (Leung & Glover, 2011; Reinhardt & Yaffe, 2013). Classically, BRCT domains are associated in tandem in a tight head-to-tail fashion through a large hydrophobic interface over which they bind the pSxxF recognition motif of partner proteins through two main interactions. The phosphorylated serine is anchored through electrostatic interactions to the first BRCT domain, while the phenylalanine sits in a hydrophobic cleft of the second BRCT domain (Manke *et al*, 2003; Shiozaki *et al*, 2004).

More recent work has revealed a much broader versatility of this protein domain, going far beyond the phospho-peptide binding mode initially assigned to BRCA1's BRCT domain. For example, the tandem BRCTs of 53BP1 are a phosphorylation-independent protein–protein interaction module that forms a heterodimer with the DNA-binding domain of p53 (Derbyshire *et al*, 2002). The MRN complex component, Nbs1, has a tandem BRCT unit that coalesces with its FHA domain to bind a diphosphorylated pSer-Asp-pThr-Asp motif in Mdc1 (Lloyd *et al*, 2009), and multiple BRCT domains are present as single structural unit in TOPBP1 (Dbp11 in *S. cerevisiae*, Rad4 in *Schizosaccharomyces pombe*; Rappas *et al*, 2011). Intriguingly, individual BRCT domains in TOPBP1 bind a combinatorially diverse set of phospho-ligands of varying sequences that appear to adopt conformationally similar binding modes (Day *et al*, 2018). In other cases, single BRCT domains are used as adaptors to mediate binding in a phosphorylation-independent manner, as in the case of the XRCC1-DNA ligase III interaction or the budding yeast translesion synthesis DNA polymerase Rev1 interaction with PCNA (Dulic *et al*, 2001; Pustovalova *et al*, 2013). Finally, BRCT domains can also contact DNA, as in the case of human replication factor C p140 (Kobayashi *et al*, 2010), or the XRCC1 BRCT1 domain, which binds both poly(ADP-ribose) and DNA using two non-overlapping regions (Polo *et al*, 2019).

The H-BRCT variant found in Sir4 contains an N-terminal α-helix attached to the BRCT core, that was first identified in the Dbf4

Table 1. Binding motifs of phospho-peptides.

	−2	−1		+1	+2	+3	K_D (Sir4 H-BRCT) [μM]	K_D (Dbf4 H-BRCT) [μM]
Esc1^{pS1450}	L	P	**pS**	D	P	P	0.07	0.90
Ubp10^{pT123}	L	S	**pT**	E	P	P	0.07	0.63
Ty5^{pS1095}	L	D	S	**pS**	P	P	5.57	n.d.
Tom1^{pS2376}	I	D	**pS**	D	F	L	35.06	75.46
Cbf1^{pS155}	M	T	**pS**	S	P	M	15.96	172.43

Consensus: [LIM]-x-[ST]-[SDE]-[PF]-[PLM].
Table listing interacting protein residues around the phospho-group (shown in bold) of phospho-peptides for which K_D values, from MST measurements, for Sir4 H-BRCT and Dbf4 H-BRCT binding were determined. A consensus motif is listed below the table.

regulatory subunit of the Dbf4-dependent kinase (DDK, Dbf4-Cdc7), where it is used to bind the FHA1 domain of Rad53 in a phosphory-lation-independent manner (Matthews *et al*, 2012, 2014; Almawi *et al*, 2016). Here, we show that Dbf4 H-BRCT also has the capacity to bind to phosphorylated Esc1 and Ubp10 peptides, with nanomo-lar affinity, as does Sir4 H-BRCT. The Dbf4 H-BRCT-Ubp10 pT123 peptide binding may be physiologically relevant, given that Ubp10 stabilizes Dbf4 levels through deubiquitination in order to activate the Cdc7 kinase, allowing Cdc7-Dbf4 to phosphorylate the MCM helicase and catalyze the initiation of DNA replication at the G1/S transition (Mapa *et al*, 2018). This is further corroborated by the finding that deletion of the N-terminal intrinsically disordered region of Ubp10, which contains the Ubp10^{pT123} peptide, leads to a reduc-tion of Dbf4 levels and delays S-phase entry (Mapa *et al*, 2018). Further work is needed to determine whether the Dbf4 H-BRCT domain acts as versatile phospho-protein binder, as demonstrated for the Sir4 H-BRCT domain. Intriguingly, given that the putative phospho-epitope binding region in Dbf4 H-BRCT partially overlaps with the surface patch engaged in FHA1-Rad53 interaction (Almawi *et al*, 2016), the two binding modes may be mutually exclusive.

We have characterized biochemically and structurally the Sir4 H-BRCT domain and have shown that it is critical for Sir4's silencing function. Mutations in the Sir4 H-BRCT domain that disrupt phospho-epitope binding result in mislocalization of Sir4, most likely due to the loss of Sir4-Esc1 interaction, and loss of heterochromatic repression. The link to ubiquitin ligases through Sir4 may also contribute to heterochromatin formation and the nuclear localization of silent domains (Burgess *et al*, 2012; Gard-ner *et al*, 2005).

Our structural and biochemical data of Sir4 H-BRCT ligands suggest that an accessible phosphorylated sequence stretch contain-ing the PROSITE string [LIM]-x-[ST]-[SDE]-[PF]-[PLM], where S/T are phosphorylated, characterizes interactors of the Sir4 H-BRCT (Table 1). Hydrophobic residues at positions −2 and +2, +3 with respect to the phosphorylation site, are required for interaction with indole rings of the double tryptophan motif and a double proline at +2, +3 positions results in tightest binding. Side chains of residues at position −1 point into solvent and contribute to binding only through their backbone atoms. Therefore, this position can probably be populated by any amino acid. The serine- or threonine-linked phospho-group interacts with the Arg1066, Trp1068, and Lys1072 triad while the +1 glutamate side chain in the case of Ubp10^{pT123} is long enough to additionally engage Arg1075 in an electrostatic

interaction. Ty5^{pS1095} also profits from Arg1075 and binds its guani-dinium group at the +1 phosphorylated serine residue. Having the phospho-group shifted toward the C-terminus by one residue (+1 position) results in approximately tenfold lower affinity.

Based on measured affinity constants for peptides of varying sequences, structural insights, and the observation that a phos-pho-site at position +1 disfavors tight interaction, we defined an idealized consensus motif, [LI]-x-[ST]-[DE]-P-P, which is predicted to bind either Sir4 H-BRCT or Dbf4 H-BRCT. Using this consen-sus, we carried out a ScanProsite (https://prosite.expasy.org/scan prosite/) search against the yeast nuclear proteome (Gauci *et al*, 2009). The results (presented in Table EV5) show that the motif was found in just one protein, Mcm6, besides the well-character-ized Sir4 interactors Esc1 and Ubp10. Mcm6 is a subunit of the heptameric minichromosome maintenance complex (MCM), and multiple MCM subunits are targets of the Dbf4-dependent kinase DDK (Larasati & Duncker, 2016), which is critical for the initia-tion of DNA replication. DDK docking sites have been mapped to MCM subunits 2/4, but our data suggest that the phospho-motif on Mcm6 (875-ITSEPP-880), located on a disordered stretch of the C-terminus, may contribute to DDK recruitment. Further investigations will be needed to validate this putative DDK dock-ing site on the MCM complex.

A less stringent search string that allowed a phospho-site at posi-tion +1 and an additional hydrophobic residue of similar length at position −2, [LIM]-x-[STDE]-[STDE]-P-P, detected Ty5 and three additional proteins (Bir1, Taf10, and Tod6), which have not been characterized as Sir4 or Dbf4 interactors to date (Table EV5). Hits in the lower stringency search require biochemical and genetic analy-sis to establish whether or not these putative ligands have biological relevance.

Here, we report micromolar affinity of the Sir4 H-BRCT domain for a phosphorylated motif within the E3 ubiquitin ligase Tom1. Tom1 is required for cell cycle-dependent ubiquitination and degra-dation of Dia2, a component of the SCF^{Dia2} E3 ubiquitin ligase involved in DNA replication (Kim & Koepp, 2012). Interestingly, Dia2 ubiquitylates Sir4 and cells lacking Dia2 show silencing defects in yeast heterochromatin (Burgess *et al*, 2012). Therefore, it is conceivable that Tom1 and Dia2 ubiquitin ligases are part of a cell cycle-dependent auto-regulatory loop that controls Sir4 levels. In short, we speculate that in the absence of phosphorylated Tom1, Dia2 is able to ubiquitylate Sir4, leading to its degradation. However, when Tom1 is phosphorylated and interacts with Sir4, it

may be able to ubiquitylate and degrade Dia2, thereby antagonizing the degradation of Sir4. It is well established that Sir4 dosage levels need to be tightly controlled in order to ensure gene silencing, as a single extra copy of *SIR4* disrupts silent chromatin (Cockell *et al*, 1995; Smith *et al*, 1998). It is possible that the controlled recruitment of Tom1 by Sir4 contributes to this homeostasis.

Heterochromatin assembly and disassembly is a dynamic cell cycle-dependent process regulated by phosphorylation and ubiquitylation events (Emre & Berger, 2006; Weake & Workman, 2008). An important conclusion from our study is that there is a non-canonical phospho-epitope binding H-BRCT domain in the heterochromatin scaffolding protein Sir4, which provides a platform for binding phosphorylated proteins. The phospho-epitope binding motif within Sir4 is shown to be essential for stable heterochromatin formation and for the sequestration of Sir4-tagged heterochromatic foci at the nuclear periphery.

Materials and Methods

Cloning, expression, and purification of H-BRCT and FHA1 domains

Sir4 H-BRCT Se-Met and native versions

The region corresponding to aa 961–1,085 in UniProt entry P11978 from *S. cerevisiae* Sir4 was subcloned into pOPINF using InFusion cloning (Berrow *et al*, 2007) to yield an N-terminal His$_6$ tag with a 3C cleavage site between the tag and the protein of interest. Native Sir4 H-BRCT expression was performed in *E. coli* BL21 (DE3) Star cells through auto-induction for 20 h at 18°C. In the case of Se-Met derived Sir4 H-BRCT protein, expression was carried out in *E. coli* B834 (DE3) cells (Merck) induced by 0.5 mM IPTG in selenomethionine medium (Molecular Dimensions, UK) for 20 h at 20°C. Cell pellets were harvested and resuspended in nickel lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM TCEP, 2.5 mM MgCl$_2$, 0.2% Tween-20), frozen on dry ice, and then stored at −80°C. The thawed cell suspension was diluted with cold nickel lysis buffer, freshly supplemented with Benzonase (Sigma) and Complete EDTA-free protease inhibitors (Roche), then filtered through Miracloth (Merck), and passed two times through the EmulsiFlex-C3 cell disruptor (Avestin Europe, GmbH) at 15,000 psi. The total cell lysate was centrifuged at 35,000 × *g* for 30 min at 4°C, and the soluble lysate was collected and filtered through a Minisart MNL Plus filter (Sartorius) prior to incubation in batch mode with Ni-NTA Superflow beads (Qiagen). The Ni-NTA beads were transferred into a Bio-Rad Econo column and washed with low-salt nickel wash buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM TCEP), then with the same plus 500 mM NaCl, and again with 150 mN NaCl. Digestion with 1 mg His-tagged 3C protease was performed overnight at 4°C. Target protein was collected in the column flow-through and wash fractions, concentrated over an Amicon 10 kDa MWCO Ultra device to 4 ml, and separated on a gel-filtration S75 HiLoad column pre-equilibrated in GF buffer (20 mM Tris-Cl, pH 7.4, 200 mM NaCl, 2 mM TCEP). Peak fractions containing the target protein were pooled and concentrated to 1.91 mM (native wt), 4.57 mM (native RKR mutant), and 1.13 mM (Se-Met), and aliquots were snap-frozen in N$_2$(liq) and stored at −80°C.

Dbf4 H-BRCT

The region corresponding to aa 99–250 in UniProt P32325 from *S. cerevisiae* Dbf4 was subcloned into pOPINF by InFusion cloning (Berrow *et al*, 2007), yielding an N-terminal His$_6$ tag with a 3C cleavage site followed by the H-BRCT domain (Matthews *et al*, 2012). A C-terminal extension of ~ 35 aa enhanced protein expression (Matthews *et al*, 2009). Dbf4 H-BRCT expression, purification, and storage were performed as described for the Sir4 H-BRCT domain, except that Tris buffers were at pH 8.0, and Dbf4 H-BRCT protein concentrates to 1.2 mM.

A construct encoding aa 29–158 of *S. cerevisiae* serine/threonine-protein kinase Rad53 (UniProt P22216), encompassing the FHA1 domain, was subcloned into pOPINF using InFusion cloning, generating an N-terminal His6 tag and 3C cleavage site upstream of FHA1. Expression and purification were essentially as described for the H-BRCT domains. Following the Ni-NTA affinity column, an S75 HiLoad column was used, and purified His-tagged Rad53 was digested with 3C protease and passed over a Ni-NTA column, collected in the flow-through and first wash fractions. Purified Rad53 was concentrated over a 3 kDa MWCO Amicon filter, separated by gel filtration on a S75 HiLoad column, and concentrated in running buffer (20 mM Tris, pH 7.4, 200 mM NaCl, 1 mM TCEP, 0.02% NaN$_3$) to 3.15 mg/ml (tagged) and 1.33 mg/ml (non-tagged), respectively.

Crystallization

Crystallization was carried out using the sitting-drop vapor diffusion method at 20°C with a Phoenix nano-liter dispensing robot (Art Robbins). Unbound Se-Met or native Sir4 H-BRCT crystals were obtained by mixing 200 nl protein at approximately 1 mM in 20 mM Tris, pH 7.5, 200 mM NaCl, 0.5 mM TCEP, 0.02% NaN$_3$, with 100 nl Silver Bullet (condition F1, Hampton Research) and 100 nl crystallization buffer (35% PEG 2000 MME, 150 mM potassium bromide). Silver Bullet condition F1 consists of 0.25% (w/v) methylenediphosphonic acid, 0.25% (w/v) phytic acid sodium salt hydrate, 0.25% (w/v) sodium pyrophosphate tetrabasic decahydrate, 0.25% (w/v) sodium triphosphate pentabasic, 0.02 M HEPES sodium, pH 6.8. Plates were incubated at 4°C for 10 days; crystals were cryoprotected under crystallization conditions with 20 mM Tris, pH 7.5, and 15% ethylene glycol followed by cryo-cooling in N$_2$(liq).

Sir4 H-BRCT-Esc1^{pS1450} peptide co-crystals were obtained by mixing 100 μl of protein solution containing 1.0 mM Se-Met Sir4 H-BRCT and 1.36 mM Esc1^{pS1450} phospho-peptide (aa 1,443–1,458: IPSTDLP**pS**DPPSDKEE, Peptide Specialty Laboratories GmbH, Germany) in 0.02 M Tris pH 7.5, 0.2 M NaCl, 0.002 M TCEP, 0.02% NaN$_3$ with 100 μl of crystallization buffer containing 30% PEG4000, 0.2 M ammonium acetate, and 0.1 M Na-acetate pH 4.6. Rod-like crystals were cryo-protected in crystallization buffer with 20% ethylene glycol and 1.36 mM Esc1^{pS1450} phospho-peptide followed by cryo-cooling in N$_2$(liq).

Sir4 H-BRCT Ty5^{pS1095} peptide co-crystals were obtained by mixing 100 μl of 1.0 mM Se-Met Sir4 H-BRCT and 2.7 mM Ty5^{pS1095} phospho-peptide (aa 1,087–1,103: ESPPSLDS**pS**PPNTSFNA, Peptide Specialty Laboratories GmbH, Germany) in 0.02 M Tris pH 7.5, 0.2 M NaCl, 0.002 M TCEP, 0.02% NaN$_3$ with 100 μl of crystallization buffer containing 2.0 M (NH$_4$)$_2$SO$_4$, 0.2 M Na-potassium tartrate, and 0.1 M Na$_3$-citrate pH 5.6. Thin rod-like crystals were

cryo-protected in crystallization buffer with 3.2 M $(NH_4)_2SO_4$ and 2.7 mM Ty5^{pS1095} phospho-peptide followed by cryo-cooling in $N_2(liq)$.

Sir4 H-BRCT-Ubp10^{pT123} peptide co-crystals were obtained by mixing 100 nl of 1 mM native Sir4 H-BRCT protein with a 1.3 mM Ubp10^{pT123} phospho-peptide (aa 117–128: LSTEL**pT**EPPSS, Peptide Specialty Laboratories GmbH, Germany) in 20 mM Tris, pH 7.5, 200 mM NaCl, 1 mM TCEP with 100 nl crystallization buffer containing 27.8% PEG 4000, 0.1 M Na-acetate, pH 4.9, 0.2 M ammonium acetate, and 3% w/v trimethylamine N-oxide dehydrate. Rod-like crystals were cryo-protected under crystallization conditions with 1 mM Ubp10^{pT123} phospho-peptide and 13% ethylene glycol followed by cryo-cooling in $N_2(liq)$.

Data collection, structure solution, and refinement

X-ray data collection was performed at SLS PX-II/III beamlines in Villigen, Switzerland, at 100 K. Diffraction images were processed and scaled using the XDS package (Kabsch, 2010). The structure of Sir4$^{(961–1,085)}$ was solved by the single anomalous diffraction method (SAD) using HKL2MAP (Pape & Schneider, 2004) with data collected at the peak wavelength ($\lambda = 0.97903$ Å) of a selenomethionine derivative Sir4 H-BRCT crystal (C2 space group with one molecule in the asymmetric unit). Four selenium sites were located with SHELXD and used for phasing and density modification in SHELXE (Sheldrick, 2008), followed by automated model building in BUCCANEER (Cowtan, 2006). Models were manually completed in COOT (Emsley et al, 2010) and refined in PHENIX (Adams et al, 2010) and used for molecular replacement against a native data set later collected at 1.1 Å resolution. Further refinement used alternating cycles of manual rebuilding in COOT and refinement in PHENIX using anisotropic B-factor methods and explicit hydrogens.

Sir4 H-BRCT peptide co-crystal structures with Esc1^{pS1450} (2.5 Å), Ty5^{pS1095} (3.0 Å), and Ubp10^{pT123} (2.18 Å) were solved by molecular replacement using the native Sir4 H-BRCT structure as search model with PHASER (McCoy et al, 2007). The Sir4 H-BRCT-Esc1^{pS1450} peptide complex crystallized in space group P1 with eight copies of the complex in the unit cell. Positive mF_o-DF_c difference electron density for the missing peptide allowed model building in all copies of Sir4 H-BRCT. The Sir4 H-BRCT-Ty5^{pS1095} peptide complex crystallized in space group $P4_12_12$ with three copies of the complex in the asymmetric unit. Positive mF_o-DF_c difference electron density for the missing peptide was clearly visible and allowed model building in all Sir4 H-BRCT chains. The Sir4 H-BRCT-Ubp10^{pT123} peptide crystals were space group $P2_1$ with seven peptide complexes in the asymmetric unit. Phospho-peptides were built into good quality, positive mF_o-DF_c difference electron density in all chains. Final structures for all peptide complexes were obtained by alternating rounds of manual rebuilding in COOT followed by structural refinement in PHENIX and Buster (Bricogne et al, 2010). Final structures were validated using MolProbity (Chen et al, 2010) and COOT. Structural images for figures were prepared with PyMOL (http://pymol.sourceforge.net/).

Microscale thermophoresis

N-terminal Cy5-labeled peptides (Table EV3 for the sequences of peptides used) were synthesized by Peptide Specialty Laboratories

GmbH, Heidelberg, Germany. The peptides were prepared as 5 mM stocks in 0.1 M Tris, pH 8.0 and diluted to 100 nM in MST diluent (20 mM Tris, pH 7.5, 200 mM NaCl, 1 mM TCEP, 1% BSA, and 0.1% Tween-20). A twofold dilution series of Sir4 H-BRCT or Dbf4 H-BRCT protein was prepared in MST diluent and separately mixed 1:1 with Cy5-labeled peptide to give a fixed final concentration of 50 nM Cy5-labeled peptide. To study the effect of sulfate ions on H-BRCT binding to Ty5 peptides, 200 mM Na_2SO_4 was used in place of the 200 mM NaCl in the MST diluent. The mixtures were incubated for 15 min in the dark and then loaded into premium capillaries and measured in a Monolith NT.115 (NanoTemper Technologies GmbH, Munich, Germany). The instrument settings were 25°C, 20% MST power, 20% LED power and nano-red with fluorescence before 5 s, MST on 30 s, fluorescence after 5- and 25-s delay. Measurements were performed using NT control software v2.1.31, and experiments were repeated independently ≥ 3 times. Data were analyzed using MO Affinity Analysis 2.3 (NanoTemper Technologies GmbH) using the signal from thermophoresis to calculate the binding affinities with the K_D fit model for a molecular interaction with a 1:1 stoichiometry.

Thermofluor assay

SyproOrange (Sigma) was diluted 1,000-fold in 1.2 ml of sample buffer (20 mM Tris–HCl, pH 7.5, 300 mM NaCl, 1 mM TCEP). Twenty microliters of 10 mg/ml of wt or mutant RKR Sir4 H-BRCT was added to the diluted SyproOrange solution. Twenty-four different buffers were selected from the SlicepH screen (rows F-G from Hampton Research) and were diluted to 500 mM, and 10 μl was dispensed in quadruplet into 96-well PCR plates (Thermo, AB-700). 10 μl of the protein-SyproOrange solution was mixed with the 10 μl buffer in the PCR plate, and the plate was sealed with iCycler optical tape (Bio-Rad) and centrifuged 500 × g for 1 min. Fluorescence was measured in a StepOne Plus (Applied Biosystems) real-time PCR machine. Data acquisition was done within the StepOne Plus software, running a melt curve experiment with ROX as the selected reporter, while the quencher and the passive reporter were set to zero. The temperature gradient started at 25°C, with increments of 1°C per minute, to 95°C. After each run, the experiments were analyzed and saved with the StepOne Plus software and then exported into Protein Thermal Shift software v1.3 (Applied Biosystems) to generate the mean of the quadruplet melting point derivatives. Each experiment was repeated three times for each domain. Figures were generated using GraphPad v8.1.

SEC-MALS of H-BRCT domains

Quality and oligomeric status of purified H-BRCT domains were analyzed by size exclusion and multi-angle light scattering. Purified H-BRCT domains at 10 mg/ml or monomer BSA (Sigma) at 2 mg/ml were filtered through a 0.1-μM Amicon filter before injecting 38 μl onto a Superose 6 Increase 10/300 GL column (GE Healthcare) connected to an Agilent 1100 HPLC. Separation was carried out at 0.5 ml/min in 20 mM Tris, pH 7.5, 200 mM NaCl, and 1 mM TCEP. The UV, light-scattering miniDAWN TREOS, and refractive index Optilab T-rEx detectors (Wyatt Technology) were connected in series. Data analysis used the Zimm fitting method in Astra 6.1.1 software (Wyatt Technology), and figures were generated using GraphPad v8.1.

SEC-MALS for Rad53 and Sir4 H-BRCT interaction

For Rad53 FHA1 and Sir4 H-BRCT interactions, untagged Rad53 FHA1 (1 mg/ml, 68 μM) was incubated alone or with untagged Sir4 H-BRCT (4.4 mg/ml, 296 μM) on ice for 3 h, and samples were filtered through an 0.1-μm Amicon filter and 100 μl was injected onto a Wyatt SEC 300 Å column coupled to an AKTA Basic FPLC connected in series to a light-scattering miniDAWN TREOS and refractive index Optilab T-rEx detectors (Wyatt Technology). Separations were at 4°C, in 20 mM Tris-Cl, pH 7.4, 200 mM NaCl, 1 mM TCEP, 0.02% NaN$_3$ at flow rate 0.5 ml/min and were analyzed by the Zimm fitting method in Astra 6.1.1 software (Wyatt Technology). Figures were generated using GraphPad v8.1

Yeast strains

All *S. cerevisiae* strains are described in Table EV4. Cells were grown using standard techniques (30°C in YPAD medium) for yeast strain generation and growth unless otherwise stated. For competition experiments, overexpression of Sir4 H-BRCT domain (either wt or carrying R1066A, K1072A, R1075A mutations) was achieved in yeast cells (Fig 4A: GA-10372, Fig 4D and E: GA-1981) by expressing Sir4 aa 961–1,085 in-frame with a nuclear localization signal at the N-terminus and a Myc-tag at the C-terminus from plasmid OGS3459 (Sigma).

Silencing assays

Silencing of indicated reporter genes was performed as described (Gotta *et al*, 1998) after growth overnight in selective media. Five-fold dilution series starting at 10^7 cells/ml were performed in triplicates on appropriate media.

Fluorescence microscopy

Cells were grown in synthetic complete (SC) media with 4× additional adenine (72 mg/l) to prevent auto-fluorescence. Cells were fixed in fresh paraformaldehyde (PFA) 4% w/v for 5 min, washed six times in PBS, and then attached to a 0.17-mm glass coverslip using Concanavalin A. Images were captured on a Metamorph-driven Spinning-disk confocal system based on Olympus IX81 microscope equipped with a Yokogawa CSU-X1 scan head, EM-CCD cascade II (Photometrics) camera, and a ASI MS-2000 Z-piezo stage. We used a PlanApo ×100, NA 1.45 total internal reflectance fluorescence microscope oil objective and VisiView software. Fluorophores were excited at 561 nm (for RFP) and 491 nm (for GFP), and emitted fluorescence was acquired simultaneously on separate cameras. Image analysis was performed with NIH ImageJ.

Immunoprecipitation and Western blotting

Immunoprecipitation was performed as in Deshpande *et al* (2017) with the following modifications: Myc-Trap (chromotek-ytma-20) magnetic beads were used to pull-down Sir4-Myc. Roche complete protease inhibitor tablets (Sigma-04693116001) were used at twice the recommended concentration to prevent Sir4/Tom1 degradation. Bead washing was performed in Lambda protein phosphatase buffer with or without 2,000 units of Lambda protein phosphatase

(NEB-P0753S). Samples were incubated for 30 min at 30°C and resuspended in Laemmli buffer. The following antibodies were used: anti-HA antibody (Santa Cruz sc-7392), anti-Tubulin antibody (Thermo Fisher Scientific MA1-8817), and anti-Myc antibody (in-house antibody service of the Friedrich Miescher Institute for Biomedical Research).

Affinity purification for LC-MS/MS

Yeast cells (GA-1981) were transformed with the overexpression plasmid OGS3459 containing Sir4 H-BRCT either wt or with R1066A, K1072A, and R1075A. 50 ml of log-phase yeast cultures (OD$_{600}$ = 0.5) was harvested and washed once with ice-cold phosphate-buffered saline in the presence of COmplete Protease Inhibitor Cocktail and PhosSTOP Phosphatase Inhibitor Cocktail (Roche). Cells were snap-frozen in N$_2$(liq). Thawed pellets were lysed in lysis buffer [50 mM HEPES pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% NP-40, COmplete Protease Inhibitor Cocktail, PhosSTOP Phosphatase Inhibitor Cocktail (Roche)], by bead-beating with Zirconia beads. Lysate was spun at 12,000 *g*, 10 min, 4°C, and the supernatant was filtered through a 0.45-μm centrifugal filter. The cleared lysate was incubated with 8 μl of Myc-Trap_MA beads (ChromoTek) at 4°C for 4 h. Beads were washed with lysis buffer lacking NP-40 and protease inhibitor cocktail. The enriched proteins were digested directly on the Myc-Trap_MA beads (ChromoTek) with 0.2 μg Lys-C in 5 μl digestion buffer (3 M guanidinium chloride, 20 mM EPPS pH 8.5, 10 mM CAA, 5 mM TCEP) for 2 h at RT. Next, samples were diluted with 50 mM HEPES pH 8.5 and digested with 0.2 μg trypsin ON at 37°C, followed by 0.2 μg fresh trypsin the next day, and incubating for 5 h more at 37°C.

Mass spectrometry

The entire MS experiment was performed as biological triplicates. The generated peptides (see Affinity purification for LC-MS/MS) were acidified with TFA to a final concentration of 0.8% and loaded with 0.1% formic acid, 2% acetonitrile in H$_2$O onto a peptide trap (Acclaim PepMap 100, 75 μm × 2 cm, C18, 3 μm, 100 Å) with an EASY-nLC 1000 (Thermo Scientific). Peptides were separated on a 50 μm × 15 cm ES801 C18, 2 μm, 100 Å column (Thermo Scientific) mounted on a DPV ion source (New Objective), at a flow rate of 150 nl/min with a linear gradient of 2–6% buffer B in buffer A in 3 min followed by an linear increase from 6 to 22% in 40 min, 22–28% in 9 min, 28–36% in 8 min, and 36–80% in 1 min, and the column was finally washed for 14 min at 80% B (buffer A: 0.1% formic acid, buffer B: 0.1% formic acid in acetonitrile). The data were acquired on a Orbitrap Fusion (Thermo Scientific) mass spectrometer using 120,000 resolution for the full scan in the Orbitrap and a top T (3 s) method with HCD fragmentation for each precursor and fragment measurement in the ion trap according the recommendation of the manufacturer (Thermo Scientific).

Protein identification and relative quantification of the proteins were done with MaxQuant version 1.5.3.8 using Andromeda as search engine (Cox *et al*, 2011) and label-free quantification (LFQ; Cox *et al*, 2014) as described in Hubner *et al* (2010). The *S. cerevisiae* UniProt version 2017_04 combined with the contaminant DB from MaxQuant was searched with carbamidomethyl as a fixed modification on cysteine and variable modification oxidation on methionine, protein

N-terminus acetylation, and phosphorylation on serine, and threonine. The protein and peptide FDR were set to 0.01.

Statistical analysis was done in Perseus (version 1.5.8.5; Cox *et al*, 2011, 2014; Tyanova *et al*, 2016). Results were filtered to remove reverse hits, and contaminants and peptides found in only one sample. Missing values were imputed, and potential interactors were determined using *t*-test and visualized by a volcano plot (Fig 5B), showing only nuclear proteins as described by Gauci *et al* (2009). Significance lines corresponding to a given FDR have been determined by a permutation-based method (Tusher *et al*, 2001). Threshold values (FDR) were selected between 0.005 and 0.05 and S_0 (curve bend) between 0.1 and 2 and are shown in the corresponding figures. Results were exported from Perseus and visualized using statistical computing language R.

Data availability

The data sets produced in this study are available in the following databases:

- Atomic coordinates and structure factors: PDB 6RRV (Sir4 H-BRCT unbound; https://www.rcsb.org/structure/6rrv)
- Atomic coordinates and structure factors: PDB 6QSZ (Sir4 H-BRCT-Esc1^{pS1450} complex; https://www.rcsb.org/structure/6qsz)
- Atomic coordinates and structure factors: PDB 6QTM (Sir4 H-BRCT-Ty5^{pS1095} complex; https://www.rcsb.org/structure/6qtm)
- Atomic coordinates and structure factors: PDB 6RR0 (Sir4 H-BRCT-Ubp10^{pT123} complex; https://www.rcsb.org/structure/6rr0)
- Protein interaction MS data: PRIDE PXD014119 (http://www.ebi.ac.uk/pride/archive/projects/PXD014119)

Expanded View for this article is available online.

Author contributions

ID and HG conceived the study and designed experiments. JJK expressed, purified, and crystallized proteins. JJK and HG collected diffraction data and HG determined crystal structures. ID established yeast strains, did pull-downs for IP-MS, and performed yeast drop assays. KC did fluorescence microscopy, pull-down experiments, and drop assays. VI carried out mass spectrometry experiments and analysis. JJK performed and analyzed MST, SEC-MALS, and Thermofluor experiments. HG, ID, JJK, and SMG wrote the paper. HG and SMG supervised the work.

Conflict of interest

The authors declare that they have no conflict of interest.

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