Screen identifies DYRK1B network as mediator of transcription repression on damaged chromatin

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DNA double-strand breaks (DSBs) trigger transient pausing of nearby transcription, an emerging ATM-dependent response that suppresses chromosomal instability. We screened a chemical library designed to target the human kinome for new activities that mediate gene silencing on DSB-flanking chromatin, and have uncovered the DYRK1B kinase as an early responder to DNA damage. We showed that DYRK1B is swiftly and transiently recruited to laser-microirradiated sites, and that genetic inactivation of DYRK1B or its kinase activity attenuated DSB-induced gene silencing and led to compromised DNA repair. Notably, global transcription shutdown alleviated DNA repair defects associated with DYRK1B loss, suggesting that DYRK1B is strictly required for DSB repair on active chromatin. We also found that DYRK1B mediates transcription silencing in part via phosphorylating and enforcing DSB accumulation of the histone methyltransferase EHMT2. Together, our findings unveil the DYRK1B signaling network as a key branch of mammalian DNA damage response circuits, and establish the DYRK1B-EHMT2 axis as an effector that coordinates DSB repair on transcribed chromatin.

Significance

Cells avoid clashes between DNA repair machineries and the transcription apparatus by temporary halting gene expression in the vicinity of DNA double-strand breaks (DSBs), an emerging DNA damage response (DDR) that underlies genome integrity protection. In this study, we screened for novel activities that may be important in this DDR and have identified the DYRK1B kinase as a component of the mammalian DDR that specializes in fine-tuning DSB repair on actively transcribed chromatin. Moreover, global analysis of DYRK1B substrates has led to the identification of the histone methyltransferase EHMT2 as a DYRK1B target and effector. Our findings uncover the DYRK1B network as a DDR subpathway that preserves the integrity of active chromatin.

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The authors declare no competing interest.

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Data deposition: The phosphopeptide data generated in this study have been deposited with the ProteomeXchange Consortium via the PRIDE partner repository (PXD019102).

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of ATM in orchestrating DSB responses (24), including DSB-induced transcription silencing in cis (DISC) (6), chemical inhibitors that targeted the ATM kinase compromised DISC, as reflected by the high percentages of cells with YFP-MS2 foci (Fig. 1C, SI Appendix, Fig. S1, and Dataset S1). We filtered out kinase targets that reside in the cytoplasm (SI Appendix, Fig. S1I) and, among the list of nuclear kinase targets, we were drawn to DYRK1B (Fig. 1D). In support of the idea wherein DYRK1B may encode an ATM effector in epistatic in this DNA damage response (DDR) (Fig. 1E), we recently reported to interact with the DSB response factor RNF169 (22). We confirmed that chemical inhibition of DYRK1B, much like that of ATM, attenuated DSB-induced transcription suppression (Fig. 1D), and that nascent transcription can be inhibited by the transcription elongation inhibitor 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) (SI Appendix, Fig. S2A). In support of the idea wherein DYRK1B may encode an ATM effector in mounting transcription suppression on damaged chromatin, we found that chemical inhibition of both ATM and DYRK1B compromised DISC to the same extent as that observed in ATM inhibitor-treated cells, suggesting that ATM and DYRK1B may be epistatic in this DNA damage response (DDR) (Fig. 1E).

**DYRK1B Promotes DISC in a Kinase-Dependent Manner.** To corroborate that DYRK1B is important in transcription suppression on damaged chromatin, we depleted DYRK1B using two independent small interference RNAs (siRNAs) (SI Appendix, Fig. S2B) and found that DYRK1B silencing led to sustained transcription despite DSB induction in U2OS-DSB reporter cells (Fig. 2A). Similar observations were made in cells lentivirally transduced with two independent DYRK1B-targeting guide RNAs (gRNAs) (SI Appendix, Fig. S2 B and C). Moreover, in stark contrast to control cells, we found that DYRK1B-inactivated cells failed to suppress 5-ethyluridine (5-EU) incorporation at laser-microirradiated sites (Fig. 2B and C and SI Appendix, Fig. S2D), indicating that DYRK1B is required to inhibit nascent transcription at DNA damage sites. Given that chemical inhibition of ATM or DYRK1B attenuated suppression of 5-EU incorporation at laser-induced DSBs (SI Appendix, Fig. S2E), we genetically examined whether DYRK1B kinase activity is important in DISC by reconstituting DYRK1B-depleted cells with wild-type DYRK1B or its kinase-inactive mutants K140M and D239A (25, 26). In line with the requirement of DYRK1B kinase activity in suppressing transcription on damaged chromatin, mutational inactivation of DYRK1B catalytic activity compromised DISC in reporter cells (Fig. 2D) as well as in laser-microirradiated cells (SI Appendix, Fig. S2F). Together, these data firmly establish the DYRK1B kinase as a transcription control factor at DSBs.

**DYRK1B Promotes DISC-Associated Histone Ubiquitylation and RNAPII Dynamics.** Given that ATM mounts DISC by promoting local H2A ubiquitylation (6), we tested whether chemical inhibition of DYRK1B may similarly compromise DSB-associated histone ubiquitylation. Consistently, DYRK1B inactivation led to marked reduction in H2A ubiquitylation but did not noticeably affect levels of total ubiquitin conjugates (FK2) or K63-linked ubiquitin adducts at FokI-induced DSBs (SI Appendix, Fig. S3 A–C). Moreover, in line with a role in regulating RNAPII-dependent transcription at DSBs. (A) Schematic illustration of the DISC reporter in U2OS-DSB reporter cells. Doxycycline (Dox) induces translocation and transcription of the transgene locus. Cell treatment with 4-OHT and Shield-1 leads to FokI-induced DSBs and suppression of proximal transcription. Arrowheads denote YFP-MS2 focal points. (B) Workflow depicting the kinase inhibitor library screen using U2OS-DSB reporter cells. DSB-induced cells pretreated with each of the 760 kinase inhibitors were subjected to high-content imaging and automated analyses for YFP-MS2 focus. (C) Ranking of nuclear kinase inhibitor targets with putative roles in transcription silencing. (D) FokI-induced DSBs silence transcription in control but not in ATM- or DYRK1B-inhibited cells. Expression of FokI wild type (WT) but not its catalytically inactive mutant (D450A) suppressed doxycycline-induced transcription. Pre-treatment with ATM inhibitor (ATMi; KU55933) or DYRK1B inhibitor (DYRK1Bi; AZ191) attenuated DSB-induced transcription silencing.

![Fig. 1. Chemical screen identifies regulators of transcription at DSBs.](image-url)
transcriptional activities on damaged chromatin, DYRK1B inhibition led to sustained accumulation and phosphorylation of RNAPII at FokI-induced DSBs in U2OS-DSB reporter cells (SI Appendix, Fig. S3 D–G). Together, these findings suggest that DYRK1B may regulate transcriptional activities at DSBs in an epistatic manner with ATM.
DYRK1B Is Recruited to DSBs. We next examined whether DYRK1B may play a direct role in DSB-flanking chromatin. To this end, we tested whether DYRK1B may be mobilized in response to DNA damage. Intriguingly, we found that laser microirradiation triggered swift and transient accumulation of green fluorescent protein (GFP)-tagged DYRK1B at DNA damage tracks (Fig. 3A). To decipher how DYRK1B docks at DSBs, we generated a panel of DYRK1B deletion mutants and analyzed their ability to accumulate at laser-induced DNA damage tracks (Fig. 3B and C). Interestingly, we found a strict requirement of the DYRK1B kinase domain in supporting its accumulation at DSBs (Fig. 3D and SI Appendix, Fig. S4A), and that its kinase domain itself suffices in docking at laser-induced DNA damage tracks (Fig. 3B and E). To examine if kinase activity is required for DYRK1B DSB accumulation, we monitored the dynamic of kinase-inactive mutants (i.e., K140M and D239A) at laser-induced DNA damage tracks. In contrast to the kinase domain deletion mutant, GFP-DYRK1B K140M and D239A displayed robust accumulation at laser-microirradiated sites (SI Appendix, Fig. S4B), suggesting that DYRK1B kinase activity per se is not a prerequisite for its recruitment to DSBs.

Given that PARP plays an early role in mobilizing early-respondents to DSBs (27), we chemically inhibited PARylation using olaparib and examined whether DYRK1B accumulation at laser-induced DNA damage tracks requires PARP activity. We also pretreated cells with ATM- and ATR-specific inhibitors to explore whether these master regulators of DDRs are necessary for DYRK1B docking on damaged chromatin. Accordingly, while GFP-DYRK1B accumulation at laser-induced DSBs was only marginally attenuated in ATM/ATR-inhibited cells, PARP inhibition completely precluded DYRK1B from concentrating at DNA damage sites (Fig. 3F and SI Appendix, Fig. S4C). Inhibiting ATM and ATR, on the other hand, led to a substantial delay in GFP-53BP1 recruitment to laser-microirradiated sites (SI Appendix, Fig. S4D). Noting that DYRK1B is endowed with transcription regulatory roles on DSB-flanking chromatin, we also tested whether global transcription suppression may impact its migration to DSBs. Interestingly, chemical inhibition of transcription using a panel of small molecules that target different components of the host transcription apparatus led to quantitative attenuation of DYRK1B accumulation at DSBs (Fig. 3G and SI Appendix, Fig. S4E). These data led us to postulate that DYRK1B may preferentially target DSBs on transcribed chromatin.

DYRK1B Facilitates DSB Repair. Our observations that DYRK1B promotes transcription silencing on DSB-flanking chromatin prompted us to test whether DYRK1B kinase may be required for efficient DNA repair. To this end, we measured DNA repair kinetics following cell exposure to ionizing radiation (IR) using the comet assay. In support of a role of DYRK1B in DSB repair, IR-induced DNA damage persisted in DYRK1B-inactivated cells (SI Appendix, Fig. S5A). Moreover, expression of wild-type DYRK1B but not its kinase-inactive mutants alleviated the DNA repair defect in DYRK1B-silenced cells (Fig. 4A), highlighting a role of its kinase activity in orchestrating DSB repair processes. To consolidate a role of DYRK1B in DSB repair, we further analyzed chromosome stability by scoring chromosome breaks in DYRK1B-deficient cells following IR treatment. Consistently, we found that DYRK1B promotes repair of IR-induced chromosome breaks (SI Appendix, Fig. S5B) and that its kinase activity is similarly required for recovery from IR treatment (Fig. 4B). Together, these data implicate DYRK1B-dependent transcription silencing as a key event that fine-tunes DSB repair.

Global Transcription Shutdown Alleviates DNA Repair Defects in DYRK1B-inactivated Cells. That DYRK1B may preferentially target DSBs on transcribing chromatin (Fig. 3G and SI Appendix, Fig. S4E) and is important for efficient DNA repair led us to speculate whether the kinase may facilitate repair of DSBs within transcriptionally active chromatin. We explored this possibility by performing both the comet assay (Fig. 4C) and by scoring
Fig. 4. DYRK1B facilitates repair of IR-induced DNA damage. (A) DYRK1B-silenced cells reconstituted with Dox-inducible expression vector (TRE-Vector-Flag) or those that harbor DYRK1B alleles were induced with doxycycline. Twenty-four hours posttreatment, cells were irradiated and allowed to recover. Cells were thereafter processed for the comet assay according to standard procedures. Relative tail moment of at least 200 cells from two independent experiments was quantified using ImageJ and results were plotted. Western blotting was performed to examine the expression of DYRK1B using the indicated antibodies. The asterisk denotes the protein band that corresponds to endogenous DYRK1B. Bars represent mean ± SEM; ****P < 0.0001. (B) Representative metaphase preparations from IR-treated HeLa derivatives are shown. HeLa cells lentivirally infected with DYRK1B gRNA (DYRK1B gRNA#1) were reconstituted with vector or DYRK1B alleles. Cells were thereafter irradiated and processed to determine the number of chromosomal breaks. At least 120 metaphases from two independent experiments were counted and results were plotted. Arrowheads denote chromosome breaks. Bars represent mean ± SEM; *P < 0.05, ****P < 0.0001. (C) Scheme depicting cell processing for the neutral comet assay following transient global inhibition of transcription using DRB. (D) Western blotting was performed to evaluate the expression of DYRK1B. (E) Representative images from single-cell electrophoresis to analyze relative tail moments. Quantification is shown and was derived from at least 200 cells from two independent experiments. Bars represent mean ± SEM; ***P < 0.001, ****P < 0.0001. (F) Scheme depicting cell processing for metaphase analyses following transient global inhibition of transcription using DRB. (G) Representative metaphase preparations from IR-treated HeLa derivatives are shown. The numbers of chromosomal breaks were quantified in HeLa cells processed as in B with or without pre-DRB treatment. Bars represent mean ± SEM; *P < 0.05, **P < 0.01.
chromosome breaks (Fig. 4F) with IR-challenged cells pretreated with the transcription inhibitor DRB. Notably, global transcription shutdown not only alleviated the DNA repair defect associated with DYRK1B loss (Fig. 4D and E) but also suppressed chromosome breaks in otherwise DYRK1B-inactivated cells (Fig. 4G). Together, these data suggest that DYRK1B may facilitate repair of DSBs by orchestrating local transcriptional activities.

**Phosphoanalysis of DYRK1B Substrates Identifies DISC Factors.** Given the requirement of DYRK1B kinase activity in orchestrating DSB-induced transcription silencing and in DNA repair, we performed a global profiling of DYRK1B targets with the aim to delineate how DYRK1B mediates these DSB responses (Fig. 5A). We sampled and enriched phosphopeptides obtained from parental RPE-1 cells as well as DYRK1B-overexpressing and DYRK1B-inactivated counterparts. Accordingly, bioinformatic analyses of phosphopeptides revealed a high degree of overlap between parental RPE-1 cells and those that overexpress DYRK1B, as well as those between the two isogenic DYRK1B gRNA-targeted cell lines (SI Appendix, Fig. S6A). Notably, analysis of phosphopeptides that are significantly enriched in cells with ectopic expression of DYRK1B overexpression over its knockout counterparts (KO1+KO2) (Fig. 5B and Dataset S2) uncovered that DYRK1B preferentially targets substrates on the serine/threonine-proline (S/T-P) motif (Fig. 5C). Furthermore, Gene Ontology (GO) enrichment analysis of putative DYRK1B targets indicated that DYRK1B may be involved in a plethora of biochemical pathways, including those that regulate transcription (Fig. 5D, SI Appendix, Fig. S6B, and Dataset S3).

With a focus to decipher how DYRK1B mediates DISC, we selected a number of candidates from transcription-related pathways (Fig. 5D, SI Appendix, Fig. S6C, and Dataset S3) and performed an RNA interference (RNAi)-based validation screen to isolate novel players that are important in transcription suppression on damaged chromatin (Fig. 5E). Among those that are required for DISC, we were drawn to EHMT2 (also known as G9a), as it has established roles in gene repression (28) and represents a specialized mediator of ATM-dependent DDRs that serves to maintain genome stability of transcribed chromatin.

**EHMT2 Promotes Transcription Silencing on Damaged Chromatin.** In line with a requirement of EHMT2 in pausing transcriptional activities proximal to FokI-induced DSBs in U2OS-DSB reporter lines (Fig. 6A and 6D), genetic inactivation of EHMT2 also led to sustained 5-EU incorporation at laser-induced DNA damage tracks (Fig. 6B). Given that EHMT2 is endowed with histone lysine methyltransferase activity (28), we chemically inhibited EHMT2 using UNC0638 to examine if its catalytic activity may be required for transcription suppression on DSB-flanking chromatin (33, 34). Indeed, chemical inhibition of EHMT2 led to sustained nascent transcription at FokI-induced DSBs as well as at laser-induced DNA damage tracks (Fig. 6C and D). To further corroborate a role of EHMT2 activity in DISC, we reconstituted EHMT2-inactivated cells with either wild-type EHMT2 or its catalytic mutant (ΔSET) and assayed if its methyltransferase activity is required for transcription silencing at FokI- and laser-induced DSBs. In line with a requirement of EHMT2 activity in DISC, reexpression of wild-type EHMT2 but not its ΔSET mutant in EHMT2-inactivated cells restored transcription suppression following DSB induction (Fig. 6E and F). Together, these data establish the histone methyltransferase EHMT2 as an important activity in mounting DISC.

**DYRK1B Promotes EHMT2 Accumulation at DSBs.** To further explore how DYRK1B may effect DISC via EHMT2 phosphorylation, we first examined if EHMT2 recruitment to DSBs may depend on DYRK1B. Time-lapse imaging of GFP-EHMT2 following laser microirradiation indicated that DYRK1B enforces EHMT2 accumulation at DSBs, as GFP-EHMT2 recruitment was quantitatively attenuated in DYRK1B-inactivated cells (Fig. 7A) as well as in DYRK1B-inhibited cells (SI Appendix, Fig. S7C), although DYRK1B deficiency did not noticeably alter EHMT2 protein expression level (SI Appendix, Fig. S7D). Moreover, similar to those that underlie DYRK1B recruitment to DSBs, EHMT2 deposition at DSBs required PARP and transcriptional activities (SI Appendix, Fig. S7C). These findings prompted us to investigate whether DYRK1B may promote EHMT2 deposition at DSBs via its phosphorylation. To this end, we generated EHMT2 phosphomutants (i.e., T346A and T346D) according to the DYRK1B-enriched EHMT2 phosphopeptide (Fig. 7B and C and Dataset S3C), and examined their migration kinetics to laser-induced DSBs. Notably, while the nonphosphorylatable T346A mutation partially hampered EHMT2 recruitment to DSBs, the phosphomimicking T346D mutant accumulated with much more robust kinetics when compared with wild-type EHMT2 (Fig. 7D). These data suggest that DYRK1B facilitates EHMT2 docking at DSBs, at least in part, via targeted T346 phosphorylation.

**Discussion**

In this study, we have uncovered the DYRK1B network as a branch of mammalian DDR pathways that orchestrates transcriptional activities on damaged chromatin for effective DNA repair (Fig. 7E). By conducting a kinase inhibitor library screen, we identified DYRK1B as a S/T-P-targeting kinase that accumulates at DSBs to promote transcription suppression on DSB-flanking chromatin. Phosphoprofiling of DYRK1B substrates further unveiled that DYRK1B-dependent DISC is, at least in part, effected via EHMT2 phosphorylation and its docking at DSBs. Our findings thus highlight the DYRK1B kinase as a specialized mediator of ATM-dependent DDRs that serves to maintain genome stability of transcribed chromatin.

Our appreciation for the interplay between DSB metabolism and local transcriptional activities has grown since the introduction of elegant platforms in which DSBs can be induced at specific genomic loci (6, 7, 35–37), thus permitting the study of DSB microenvironment (e.g., histone marks) and its impact on local chromatin transactions (38, 39). Notably, it has now become evident that DSB repair can be dynamic (40, 41) and that DSB nuclear output is limited by local chromatin architecture and activities (36, 42). In particular, the molecular events that underlie transcriptional control on damaged chromatin have garnered much interest (11, 36, 42, 43), in part due to their emerging impact on genome stability and its relevance to human diseases. In this connection, seeing that DYRK1B has been associated with a metabolic syndrome (20), we subdivided the clinically derived DYRK1B mutations (i.e., H90P and R102C) and examined their impact on DYRK1B-dependent DDRs (SI Appendix, Fig. S8). Intriguingly, while both DYRK1B mutations compromised DSB repair in the comet assay (SI Appendix, Fig. S8A), R102C exhibited perturbed DSB recruitment kinetics (SI Appendix, Fig. S8B) and failed to suppress transcription following FokI- and laser-induced DSBs (SI Appendix, Fig. S8 C and D). Given the emerging links between DSB repair and metabolic homeostasis (44), it would be of significant interest to explore how defective DISC may impact cell differentiation and tissue homeostasis.

Global phosphoprofiling of DYRK1B targets led to the uncovering of the histone lysine methyltransferase EHMT2 as one of the candidate downstream effectors that promotes DSB-induced
transcription silencing (Fig. 5). Indeed, that EHMT2 plays established roles in gene repression (28) suggested that the DYRK1B–EHMT2 axis may represent a novel branch of DDRs with specialized roles in preserving transcriptionally active chromatin. In line with this notion, we found that DYRK1B targets EHMT2 at a highly conserved S/T-P motif (Fig. 7B) and that EHMT2 carrying transcription silencing (Fig. 5). Indeed, that EHMT2 plays established roles in gene repression (28) suggested that the DYRK1B–EHMT2 axis may represent a novel branch of DDRs with specialized roles in preserving transcriptionally active chromatin. In line with this notion, we found that DYRK1B targets EHMT2 at a highly conserved S/T-P motif (Fig. 7B) and that EHMT2 carrying transcription silencing (Fig. 5). Indeed, that EHMT2 plays established roles in gene repression (28) suggested that the DYRK1B–EHMT2 axis may represent a novel branch of DDRs with specialized roles in preserving transcriptionally active chromatin. In line with this notion, we found that DYRK1B targets EHMT2 at a highly conserved S/T-P motif (Fig. 7B) and that EHMT2 carrying
Fig. 6. EHMT2 promotes transcription silencing on damaged chromatin. (A) EHMT2 inactivation compromised DSB-induced transcription silencing. U2OS-DSB reporter cells lentivirally transduced with the indicated gRNAs were incubated with Dox, 4-OHT, and Shield-1 to induce DSBs proximal to the transcription unit. Thereafter, cells were processed to visualize YFP-MS2 and mCherry-FokI foci. Nuclei were counterstained with DAPI. Arrowheads denote YFP-MS2 foci. MFI of YFP-MS2 was quantified. Bars represent mean ± SEM; **** p < 0.0001. Western blotting was performed to evaluate expression of EHMT2. (B) EHMT2 silencing led to sustained nascent transcription at laser-induced DSBs. HeLa cells transduced with the indicated gRNAs were laser microirradiated. Cells were processed 1 h after to evaluate 5-EU incorporation at laser-induced DNA damage tracks. Arrowheads denote sites of laser microirradiation. Quantification of 5-EU incorporation at laser-induced γH2AX-marked DSBs was performed. Data represent mean ± SEM from three independent experiments. Western blotting was performed to evaluate expression of EHMT2. (C and D) Chemical inhibition of EHMT2 attenuated DISC. Cells pretreated with either ATM inhibitor (KU55933) or EHMT2 inhibitor (UNC0638) were processed for visualization of mCherry-FokI and YFP-MS2 (E) and laser-induced (F) DSBs. Control gRNA- or EHMT2 gRNA-targeted cells reconstituted with vector control, WT EHMT2, or its SET deletion mutant (ΔSET) were subjected to either the U2OS-DSB reporter assay as in A or the 5-EU incorporation assay as in B. Quantification and Western blotting analyses were performed as in A and B. Note that the EHMT2 antibodies were raised against a synthetic peptide corresponding to the carboxyl terminus of the protein, and do not recognize the EHMT2 SET deletion mutant.
Fig. 7. DYRK1B facilitates EHMT2 recruitment to DSBs. (A) Control (CTR gRNA) or cells transduced with DYRK1B gRNAs (DYRK1B gRNA#1 and DYRK1B gRNA#2) were transiently transfected with the GFP-EHMT2 expression construct. Twenty-four hours posttransfection, cells were microirradiated and time-lapse images were captured to monitor GFP-EHMT2 migration to laser-induced DNA damage tracks. Arrowheads denote sites of laser microirradiation. Quantification is shown and is derived from two independent experiments of at least 10 cells each. (B) Schematic illustration of EHMT2 protein domains and the DYRK1B-enriched EHMT2 phosphopeptide surrounding T346. Note that T346 corresponds to T555 in the long isoform of EHMT2. (C and D) GFP-tagged WT EHMT2 or its phosphomutants (T346A and T346D) were expressed in U2OS cells prior to laser microirradiation and time-lapse imaging experiments as done in A. (E) Working model depicting DYRK1B in orchestrating transcription suppression on DSB-flanking chromatin. DYRK1B accumulates at DSBs in a PARP-dependent manner, and promotes EHMT2 T346 phosphorylation and concentration at DSBs to effect DISC.
Materials and Methods

DYRK1B coordinates local transcription during DSB metabolism and organismal development. We await further work to decipher how it has evolved to suppress chromosomal instability and maintain DYRK1B and its network as key signaling intermediates that regulates gene expression (Fig. S11). Together, while DYRK1B likely effects transcription suppression on DSB-flanking chromatin, the fact that DYRK1B-associated DSB repair defects can be alleviated by global transcription inhibition (Fig. 4 C–F) establishes DYRK1B and its network as key signaling intermediates that promote DISC, a dedicated ATM-dependent DSB response that has evolved to suppress chromosomal instability and maintain gene expression programs important in cell proliferation and organismal development. We await further work to decipher how DYRK1B coordinates local transcription during DSB metabolism and how it translates to DSB repair and maintenance of chromosomal stability.

Materials and Methods

Cell Lines, Cell Culture, Plasmids, and Chemicals. HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin ( Gibco, Thermo Fisher Scientific) at 37 °C in 5% CO2. Details of chemicals, plasmids, and antibodies are in SI Appendix, Tables S1–S3, respectively.

RNA Interference. Cells were transfected twice at 24-h intervals with either nontargeting control or gene-specific siRNAs (Dharmacon) using Oligofectamine (Invitrogen) according to the manufacturer’s instructions. Sequences of siRNAs are listed in SI Appendix, Table S4.

Lentiviral Particle Packaging and Transduction. HEK293T cells were transiently cotransfected with lentiviral-based expression plasmids pPAX2 and pMD2.G at a ratio of 4:3:1 using polyethyleneimine to produce lentiviral particles. Forty-eight hours after transfection, supernatants containing lentiviruses were filtered with an Acrodisc 25-mm syringe filter with a 0.45-μm membrane (PALL Life Sciences) and were used for cell transduction in the presence of 8 μg/ml polybrene (Sigma-Aldrich).

Western Blotting and Coimmunoprecipitation. Cells were harvested and lysed with NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5% Nonidet P-40, 1 mM ethylenediaminetetraacetic acid [EDTA]) supplemented with benzonase nuclease (ChemCruz) for 30 min on ice. Whole-cell lysates were boiled in sodium dodecyl sulfate (SDS) loading buffer, resolved on polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidene fluoride (PVDF) membranes, and immunoblotted with the indicated antibodies. For coimmunoprecipitation, cells were lysed with NETN buffer for 30 min on ice. After centrifugation at 15,000 rpm for 15 min at 4 °C, supernatants were incubated with 200 μl streptavidin-conjugated beads (GE Healthcare, Sigma-Aldrich) for 4 h at 4 °C with gentle rotation. Protein-bound beads were washed with ice-cold NETN buffer three times and subjected to immunoblotting.

Denaturing Immunoprecipitation. HEK293T cells transiently cotransfected with Flag-EHMT2-binding peptide (SB)-tagged EHM2 mutants with myc epitope-tagged vector or myc epitope-tagged DYRK1B were lysed with denaturing buffer (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.5% Nonidet P-40, 0.5% SDS, 0.5% deoxycholate, and 1 mM EDTA) on ice for 15 min and subsequently boiled at 95 °C for 5 min. The cell lysates were cooled down on ice for 5 min and incubated with Anti-Flag Affinity Gel (Bimake.com) for 3 h at 4 °C with gentle rotation. Protein-bound beads were washed with ice-cold denaturing buffer four times and boiled with SDS/PAGE sample-loading buffer before being subjected to immunoblotting.

Immunofluorescence. Cells cultured on coverslips were processed and at the indicated time points washed with ice-cold 1× phosphate-buffered saline (PBS) twice and thereafter fixed with 3% paraformaldehyde (PFA) for 30 min at room temperature. Cells were subsequently permeabilized with 0.5% Triton X-100 for 30 s after two PBS washes. Coverslips were blocked with 5% milk before incubating with primary antibodies for 1 h at room temperature. Cells were then washed twice with PBS and incubated with secondary antibodies for 40 min. Nuclei were counterstained with DAPI for 10 s before coverslips were mounted with fluorescence mounting medium (Dako, Agilent) onto glass slides. Images were acquired on an Olympus BX51 fluorescence microscope (UPlanSApo 40×/0.95 objective).

Laser Microirradiation and Live-Cell Imaging. Laser microirradiation was carried out on an inverted two-photon microscope (LSM780; Carl Zeiss) equipped with an inverted Axio Observer.Z1 stand, motorized scanning stage, and integrated laser microbeam system, with total UV laser output set to 750 nm (8%). Cells cultured on glass-bottomed confocal dishes (SPL Life Sciences) were subjected to laser microirradiation in a temperature-controlled (37 °C) environmental chamber supplied with 5% CO2 at 24 h after transient transfection with GFP-tagged indicated plasmids. Time-lapse images were acquired by ZEN 2012 (Carl Zeiss) software with a Plan Apochromat 40×/1.4 oil differential interference contrast (DIC) M27 objective and further processed by ImageJ software to analyze mean fluorescence intensity (MFI) across the laser-microirradiated regions. MFI was quantified as the difference between the average fluorescence intensity in the laser-microirradiated regions versus the average fluorescence intensity from adjacent undamaged regions of the same size in the same nucleus.

5-Ethynyl Uridine Incorporation Assay. Nascent transcription at laser-microirradiated sites was detected by a Click-IT RNA Alexa Fluor 594 Imaging Kit (C10330; Thermo Fisher Scientific). Briefly, cells grown on glass-bottomed confocal dishes (SPL Life Sciences) for 24 h were subjected to laser microirradiation by a live Carl Zeiss LSM780 inverted confocal microscope (10× objective) with a 750-nm laser (8% output). Subsequently, cells were cultured with complete media containing 1 mM 5-EU for 1 h after laser microirradiation. 5-EU labeling was performed following manufacturer instructions. Cells were immunostained for γH2AX and nuclei were counterstained with DAPI before mounting. Images were captured by Olympus BX51 fluorescence microscope (PlanApo N 60×/1.42 oil-immersion objective). ImageJ was used to analyze relative fluorescence intensity across the laser-microirradiated stripes. An analyzing line drawn by a line tool was perpendicular to the damaged stripes, centered at the stripes with two ends at undamaged regions, and the fluorescence intensity was multiplopped. Relative fluorescence intensity was normalized to each end from the same cell.

Chromosomal Aberration Analysis. Chromosomal aberrations were analyzed by chromosome metaphase spreading. Cells were cultured in media containing 1 μg/ml colcemid (KaryoMAX Colcemid Solution in Hanks’ balanced...
salt solution; Thermo Fisher Scientific) for 3 h after 2-Gy irradiation and suspended in 0.8% sodium citrate for 15 min at 37 °C. Subsequently, freshly prepared fixative solution (methanol/acetic acid 3:1; vol/vol) was added and incubated for 5 min at 37 °C. After washing three times by the fixative solution, cells resuspended in a small volume of fixative solution were dropped onto alcohol-cleaned slides and air dried. Cells were stained with DAPI before mounting. Images were captured by a Nikon Tii-E widefield confocal microscope (100× oil-immersion objective) using MetaMorph microscopy and image analysis software (Molecular Devices).

Neutral Comet Assay. Cells were harvested and resuspended at 5 × 10⁶ cells per milliliter in ice-cold Ca²⁺/Mg²⁺-free PBS. Diluted cells were fixed with 3:1 methanol:acetic acid (LMagarose; Trevigen) at a ratio of 1:10 (vol/vol). Cell suspensions (50 μL) were pipetted onto prechilled comet slides (CometSlide; Trevigen). Slides were kept at 4 °C for 30 min and immersed in prechilled lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-Cl, 1% N-lauroylsarcosine sodium, and 1% Triton X-100) for 1 h at 4 °C followed by further immersion in freshly prepared alkaline buffer for phoresis at 60 V for 5 min. Slides were then fixed in 100% ethanol for 5 min, air dried, and stained with 1 μg mL⁻¹ propidium iodide (Sigma-Aldrich) at room temperature in the dark for 20 min. Images were captured by an Olympus BX51 fluorescence microscope (20× objective). Tail moments of comets were quantified by ImageJ software with the Open Comet plugin.

Kinase Inhibitor Library Screen. A kinase inhibitor library was purchased from Selleck Chemicals. U2OS-DSB reporter cells, a kind gift from Roger Greenberg (University of Pennsylvania, Philadelphia, PA) were used to assay transcription repression following DSB induction (23). DSBs were induced by cell pretreatment with Stealth-1 and 4-OHT, which promote the nuclear translocation of the FokI-mCherry-Lad nuclease and its docking at the LacO transgene array. Nascent transcription at the reporter gene can be monitored by local accumulation of YFP-M52 upon the addition of 1 μg mL⁻¹ doxycycline. U2OS-DSB reporter cells in 96-well microplates (PerkinElmer) were individually incubated with 10 μM kinase inhibitor for 3 h. Cells were fixed and subjected to a high-content imaging system (IN Cell Analyzer 6500HS). Dimethyl sulfoxide (DMSO) and ATM inhibitor (KU55933) were used as negative and positive controls, respectively. Data were processed by IN Carta analysis software.

Customized siRNA Screen. Customized siRNAs (three siRNAs per gene target) were purchased from GenePharma to silence selected putative DYRK1B substrates for a role in DSB-induced transcription repression. U2OS-DSB reporter cells were transfected twice at 24-h intervals with either nontargeting siRNA control or gene-specific siRNAs, and local transcription was monitored by flowthrough from the TiO₂ column was applied to a second phosphopeptide enrichment kit (the High-Select Fe-NTA Phosphopeptide Enrichment Kit; Pierce), and the eluates of both kits were dried immediately following elution to prevent loss of phosphopeptides due to the high pH. Eluates from both kits were resuspended in basic buffer A (10 mM ammonium hydroxide, pH 10) and were separated into 36 fractions on a 100 × 1.0-mm Acquity BEH C18 column ( Waters) using an UltiMate 3000 ultra high performance liquid chromatography (UHPLC) system (Thermo) with a 40-min gradient from 99:1 to 60:40 basic buffer A:8 ratio (buffer B: 10 mM ammonium hydroxide, 99.9% acetonitrile, pH 10), and then consolidated into 18 superfractions.

Multinotch MS² Analysis of TMT-Labeled Phosphopeptides. Analysis of TMT-labeled samples was carried out as previously reported (46). In-line reverse-phase fractionation with Jupiter Proteo resin (Phenomenex) was employed to further reduce the complexity of each superfracion generated by off-line bHPLC. The resin was packed into a 200 × 0.075-mm column and HPLC was carried out on a Waters nanoAcquity ultra performance liquid chromatography (UPLC). A 95-min gradient was used to elute samples from the reverse-phase resin. The gradient consisted of 97:3 to 67:33 buffer A (0.1% formic acid and 0.5% acetonitrile) to buffer B (0.1% formic acid and 99.9% acetonitrile). Electrospray ionization at 2.5 kV was used to ionize eluted peptides into an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo). Multinotch MS² reporter ion mass spectra were used to collect peptide sequence information and TMT reporter ion quantities. The Orbitrap was used to collect profile MS data over 375 to 1,500 m/z at 120,000 resolution. Collision-induced dissociation at 35.0 normalized collision energy fragmented ions for MS/MS. Centroided MS² fragment ion mass spectra data were collected in the ion trap for ions between 400 and 2,000 m/z. Up to 10 MS² ions were selected by synchronous precursor selection and fragmented by high-energy C-trap dissociation at 65.0 normalized collision energy to separate the TMT reporter ions from the precursors. Profile MS² data from 100 to 500 m/z were collected at 50,000 within the Orbitrap for quantification of TMT reporters.

Analysis of Phosphopeptides. Phosphopeptides were identified and reporter ions were quantified using MaxQuant (Max Planck Institute) with a parent ion tolerance of 3 parts per million, a fragment ion tolerance of 0.5 Da, and a reporter ion tolerance of 0.001 Da. Oxidation of methionine and STY phosphorylation were searched as variable modifications, and carbamidomethylation of cysteine residues and TMT-10plex labeling of lysine residues and N termini of peptides were set as fixed modifications. The data were searched against the human UniProt database UP000006470 (74,458 proteins and 14,562 known contaminants) known data set, including R and the packages Limma (47), ggplot2 (48), and heatmap (49). A significance criterion of Limma adjusted P value < 0.05 and a fold change of ≥ 1 were employed to produce a “significantly enriched phosphopeptide” list for each groupwise comparison. Pathway analysis was performed on the
enriched phosphopeptides for each group using the webtool Enrichr (50) and GO term complexity was reduced using REVI戈 (51).

Statistics and Reproducibility. Quantitative data represent mean ± SEM from at least three independent experiments unless otherwise noted. Two-tailed Student’s t-test was used for statistical analysis by GraphPad Prism 8. Statistical differences were considered significant at P < 0.05.

Data Availability Statement. The phosphopeptide data generated in this study have been uploaded to ProteomeXchange under accession no. PXD0019102.

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