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Ago2 facilitates Rad51 recruitment and DNA double-strand break repair by homologous recombination

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role of diRNAs in DSB repair is restricted to repair by homologous recombination (HR) and that it specifically relies
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functions directly in mediating Rad51 accumulation at DSBs. Taken together, our findings suggest that guided by
A , A 2 51 / D B .

Keywords: 51 2
(2014) **24** 532-541. 10.103 / .2014.3 25 2014

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recombination (HR). Whereas NHEJ is eff cient but er- 51
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been detected in fy cells [14]. How diRNAs facilitate re-
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by HR and specifically relies on Ago2

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cells results in a signifcant reduction in repair by HR [4].
Here we frst examined whether in humans, other Ago-

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cytometry [15]. Consistent with our previous fndings [4],
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effciency (Figure 1A, Supplementary information, Fig-
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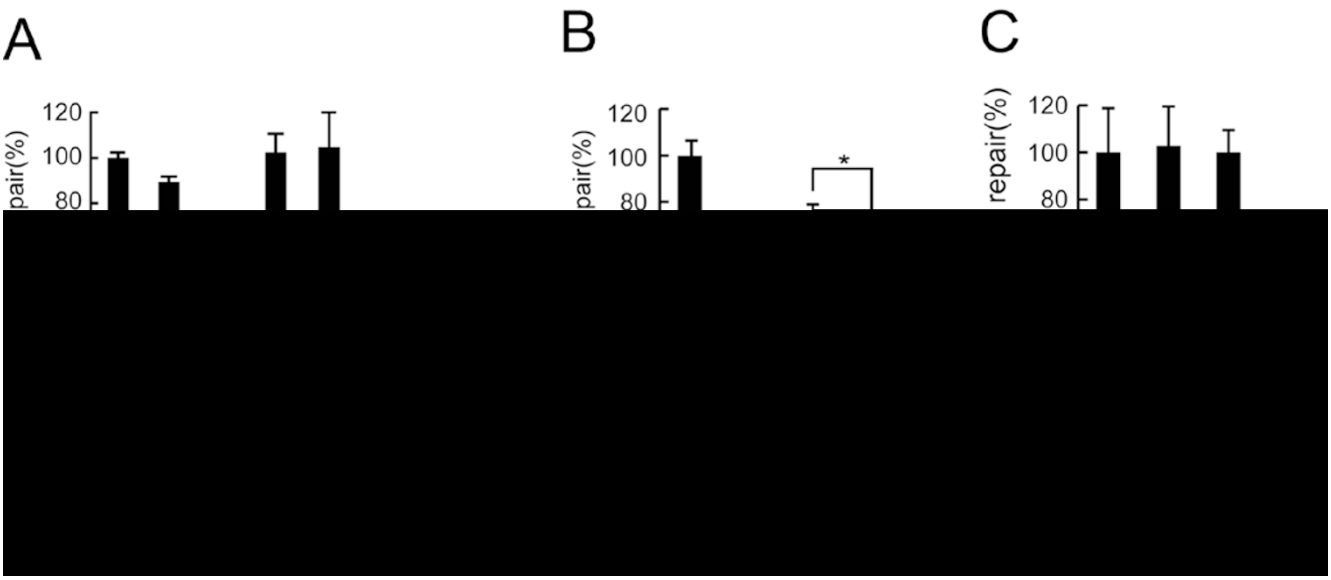

Figure 1 The role of diRNAs in DSB repair is restricted to repair by HR and specifically relies on Ago2. **(A)** HR [illegible] U2OS/DR-GFP cells treated with the indicated siRNAs. **(B)** HR repair was restored by incubation with 50 ng of sRNAs (0.25 ng/ l) prepared from U2OS/DR-GFP cells transfected with pCBA-I-*Sce*I (+I) but not empty vector (−I) in Dicer-knockdown cells. **(C)** NHEJ repair rates in HEK 293/EJ5-GFP cells treated with the indicated siRNAs. Knockdown of Ku70 that is important for NHEJ, but not Dicer or Ago2, reduced NHEJ. (*P < 0.005, **P < 0.0001, Student's t-test). The number of GFP-positive cells was measured by flow cytometry and the repair efficiency was scored as the percentage of GFP-positive cells. The extent of repair is shown relative to the repair observed in cells treated with control siRNAs. Histograms represent the mean of three independent experiments. Data are represented as mean ± SEM.

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[illegible]

The finding that Ago2 interacts with Rad51 prompted 51 - 2/ . , 51 2- - - . 2 by specific siRNAs, Rad51 but not γH2AX (the phos- 2) - (- 3 , 3 , 4) 21, 22 . 51 2 (3). , [illegible]$^{-/-}$ 23 51 - (3). 51 the previous resection of DSBs that generates 3′ over- , 51 - 2- - . , - 2 (

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2 51, that diRNAs are not necessary for efficient Ago2-Rad51 (5). , sRNA-binding deficient Ago2 mutant (Y311A/F312A or /), - 24 . - - , - / 2 51 (5), 2 51. 2 (5 , 0) 25 . ( )

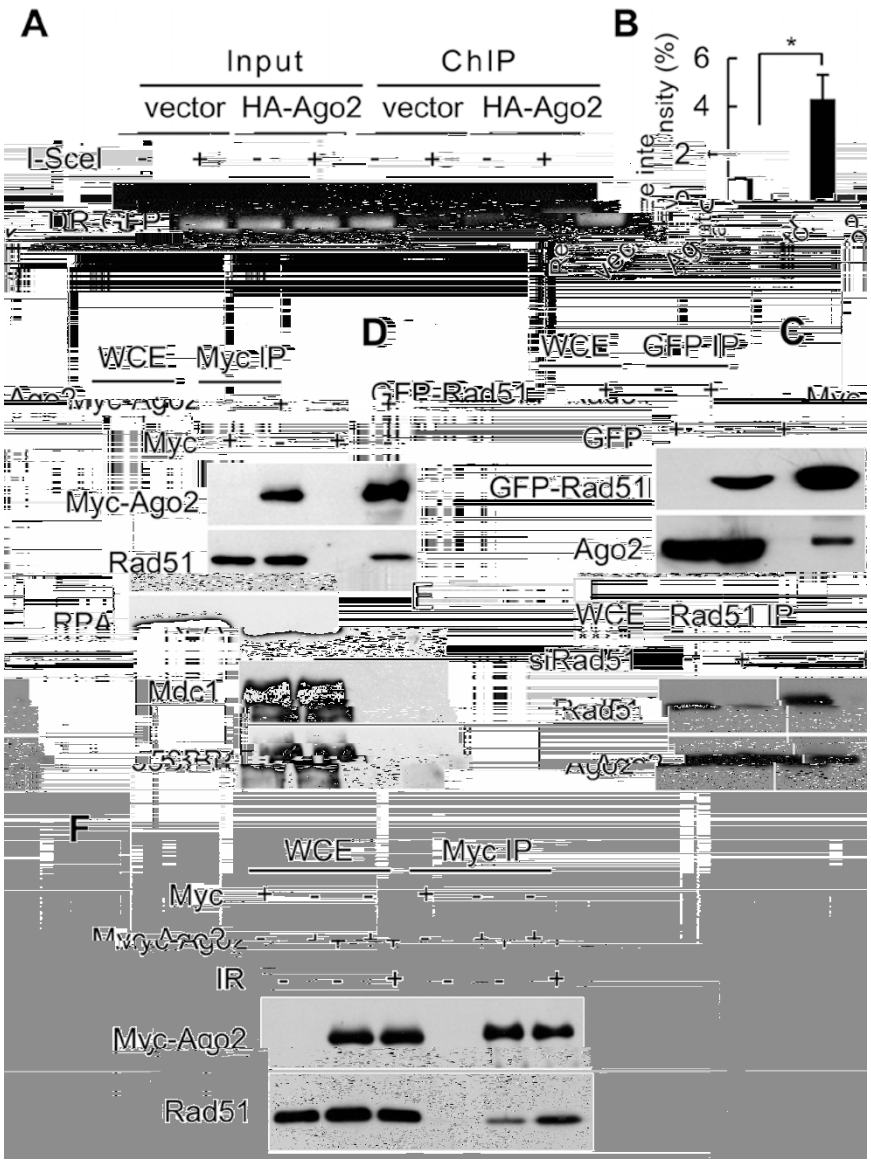

Figure 2 Ago2 accumulates at DSBs and interacts with Rad51. **(A)** U2OS/DR-GFP cells were transfected with empty vector or HA-Ago2 constructs, followed by transfection with I-*Sce*I or empty vector control. ChIP assays were performed using HA antibody. Bound DNA was analyzed by PCR. **(B)** Quantification of ChIP data shown in **A**. The quantification is based on three independent experiments. Data are represented as mean ± SEM. **(C)** Myc-Ago2 or empty vector were overexpressed in 293T cells by transfection as indicated and the lysates were subjected to immunoprecipitation using Myc-coupled beads. Immunoprecipitates (IP) and whole-cell extracts (WCE) were immunoblotted with the indicated antibodies. **(D)** GFP-Rad51 was overexpressed in 293T cells by transfection as indicated and the lysates were subjected to immunoprecipitation using GFP beads. Immunoprecipitates and WCE were immunoblotted with the indicated antibodies. **(E)** 293T cells were transfected with control or Rad51 siRNAs, lysed and the lysates were subjected to Rad51 immunoprecipitation. Immunoprecipitates and WCE were immunoblotted with the indicated antibodies. **(F)** Two days post transfection with the indicated DNA constructs, 293T cells were treated with IR (5 Gy) and left to recover for 1 h. Cells were then lysed and the lysates were subjected to immunoprecipitation using Myc-beads. Immunoprecipitates and WCE were immunoblotted with the indicated antibodies (*P < 0.005, Student's *t*-test).

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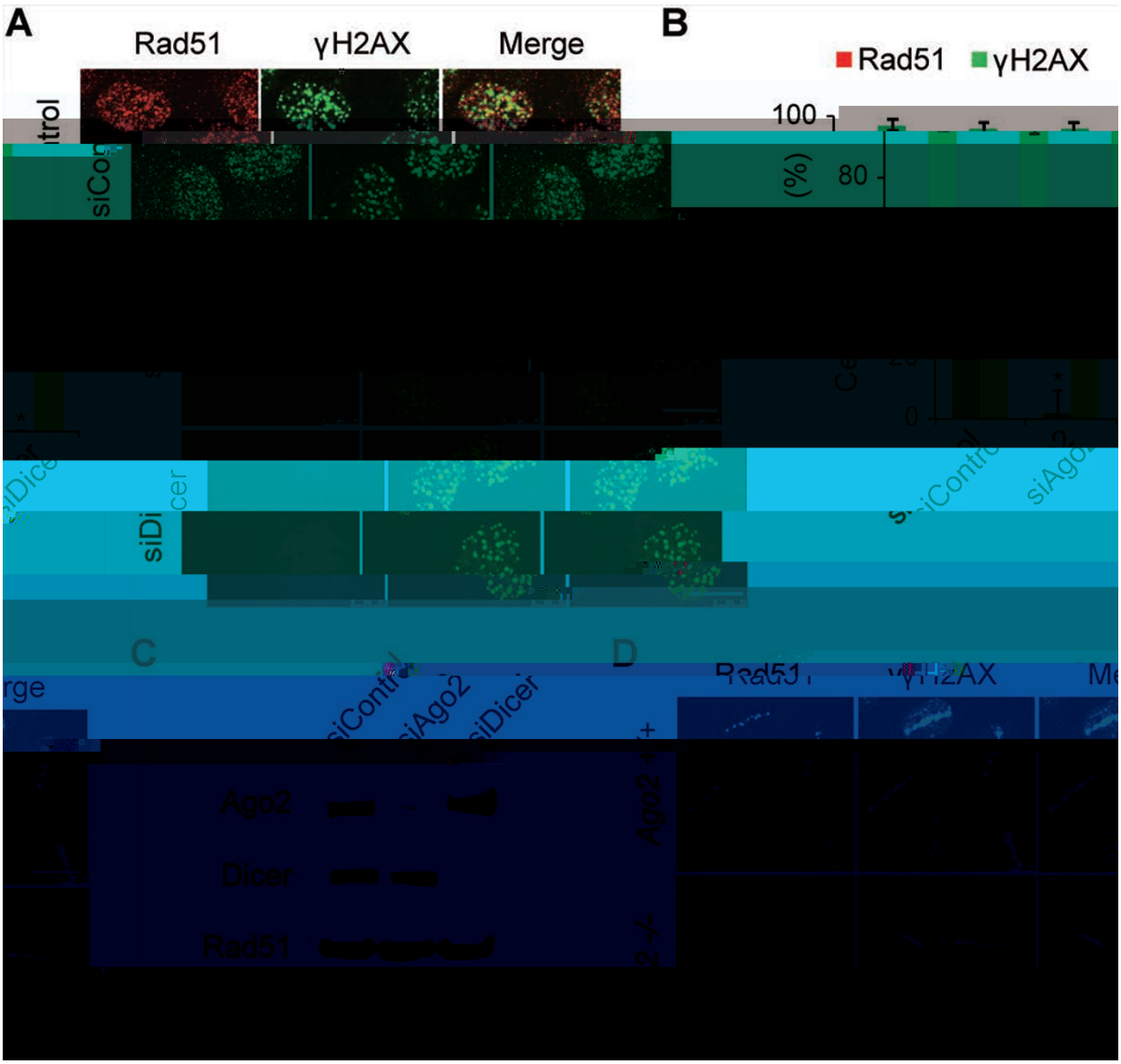

Figure 3 Ago2 and Dicer are required for Rad51 recruitment to DSBs. **(A)** U2OS cells were transfected with the indicated siRNAs for 48 h. Then, cells were treated with IR (5 Gy) and 1 h later immunostained with the indicated antibodies. Scale bars, 20 m. **(B)** Quantification of cells positive for Rad51 and γH2AX. Data from three independent experiments were used to generate the histogram. Data are represented as mean ± SEM. **(C)** U2OS cells were treated with the indicated siRNAs for 48 h, lysed and the lysates were subjected to immunoblotting with the indicated antibodies. **(D)** Wild-type or *Ago2*$^{-/-}$ MEF cells grown on microlaser dishes were treated with 10 M BrdU for 24 h. The cells were then subjected to microirradiation with pulsed UVA laser (λ = 365 nm), and 1 h later immunostained with Rad51 and γH2AX antibodies. Scale bars, 20 m. See also Supplementary information, Figure S5A and S5B. **P* < 0.005, Student's *t*-test.

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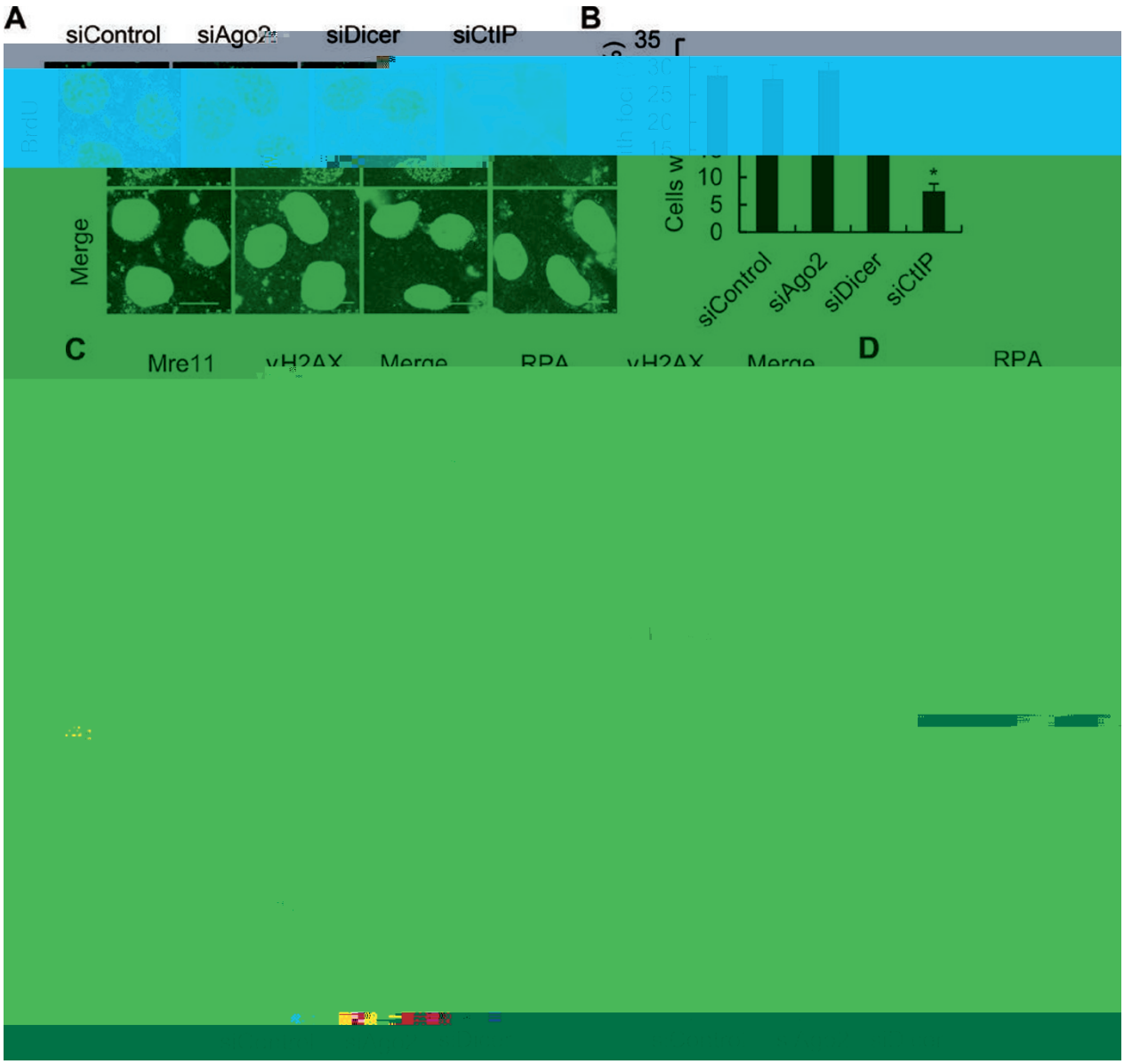

Figure 4 Ago2 and Dicer are not involved in DNA end resection or RPA/Mre11 foci formation. **(A)** U2OS cells were treated with the indicated siRNAs for 48 h and labeled with 10 M BrdU for 36 h. CPT (5 M) was then added and the cells were incubated for another 2 h. Cells were immunostained with BrdU antibody and mounted in DAPI-containing mounting medium. Scale bar, 20 M. **(B)** Quantification of BrdU foci shown in **A**. The histogram is based on data from three independent experiments. Data are represented as mean ± SEM. **(C)** U2OS cells were transfected with the indicated siRNAs for 48 h and then treated with IR (5 Gy). After 1 h, cells were immunostained with the indicated antibodies. Scale bars 20 M. **(D)** Quantification of cells with RPA and Mre11 foci shown in **C**. The histogram is based on data from three independent experiments. Data are represented as mean ± SEM. **(E-F)** Quantification of the number of RPA and Mre11 foci per cell. At least 80 cells were quantified for each condition (**P*

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Figure 5 DiRNA binding and catalytic activity of Ago2 are required for Rad51 recruitment and HR, but are dispensable for Ago2-Rad51 interaction. **(A)** 293T cells were transfected with the indicated siRNAs and DNA constructs for 48 h, lysed and the lysates were subjected to immunoprecipitation using Myc-coupled beads. Immunoprecipitates and WCE were analyzed by immunoblotting with the indicated antibodies. **(B)** Upper panel, diagram showing the Ago2 domain structure. Y311A/F312A and D669A indicate the mutations generated to produce RNA-binding defcient and catalytically inactive mutants, respectively. Lower panel, 293T cells were transfected with the indicated constructs and lysates were subjected to immunoprecipitation with HA-coupled beads. Immunoprecipitates and WCE were immunoblotted with the indicated antibodies. **(C)** Rad51 focus formation was restored in Ago2-knockdown cells by expression of siRNA-resistant wild-type Ago2 but not Y311A/F312A or D669A mutants. The histogram is based on data from three independent experiments. Data are represented as mean ± SEM. For representative pictures of immunostained cells, see Supplementary information, Figure S5A. **(D)** HR repair was restored in Ago2-knockdown cells by expression of siRNA-resistant wild-type Ago2 but not Y311A/F312A or D669A mutants. The extent of repair is shown relative to the repair observed in cells treated with control siRNAs. Data from three independent experiments were used to generate the histogram. Data are represented as mean ± SEM (*P < 0.005, Student's t-test).

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Our fnding that the catalytic activity of Ago2 is indis-

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Ago2 $^{-/-}$ MEF cells [23] were grown in Dulbecco's modifed Ea-

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DNA constructs

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was amplifed and then cloned into pMD19-T (TaKaRa) with Eco-

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and confrmed by DNA sequencing. All the Ago2 forms were then

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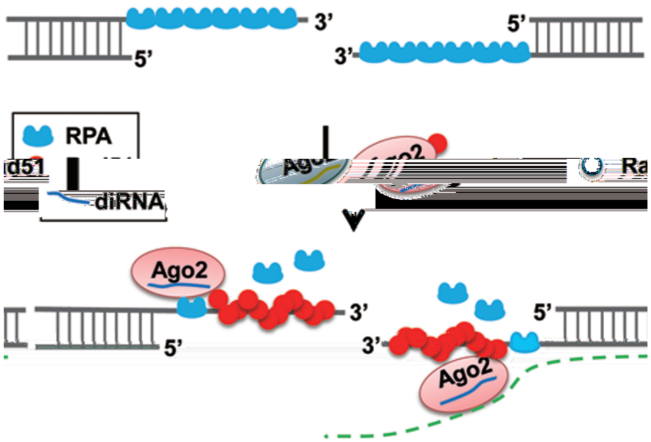

Figure 6 Working model for diRNA-directed Rad51 recruitment. Ago2-Rad51 complexes may be recruited to DNA DSB site through base pairing between diRNAs and homologous DNA sequences surrounding the break site or scaffold RNA transcripts (green dashed line) generated from DNA around the break site.

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RNAi

indicated genes: Ago1 (5'-UUCUUGAGCACCUCUU-CUCdTdT-3', 5'-CCAUUGUAGAACUGUUUCCdT-dT-3', 5'-UAUCCAGUGAGGUAACAGCdTdT-3' and 5'-AUGCUAUGAAAGUAACUCCdTdT-3') [26], Ago2 (5'-UGACAUUGGGUUCUCAUACdTdT-3') [26], Ago3 (5'- UUACCAAUCUGCUAAUUUCdTdT-3', 5'-UUGUGC-GUAAGGUAUCUUGdTdT-3', 5'-UCAUAUUGCAU-AAUGAUGCdTdT-3' and 5'-UUUGCAAAGAUAGUU-GUGCdTdT-3') [26], Ago4 (5'-AUUGCUAUUAGUUCUG-GCCdTdT-3' and 5'- UAAUAGAUGAUCCGAGUG-GdTdT-3', 5'-UCAUACUGAAAUCUCAUCUdTdT-3' and 5'-UAAGGAAGCAUCCUGGUUCdTdT-3') [26], Rad51 (5'-GGCAGUAGAUGUGCAGAUAdTdT-3', 5'-GGGACAU-GCUGCUACAAUAdTdT-3', 5'-CUGCUACAAUACGGCU-AAUdTdT-3)', Dicer (5'-UCCAGAGCUGCUUCAAGCAdT-dT-3') [29], Ku70 (5'-UGAGUGAGUAGUCAGAUCC-GUdTdT-3') [30], CtIP (5'-UAGUUUUGUCCAAAGGU-CCdTdT-3' and 5'-GAUUCGUUCCUGUUUUAGCdTdT-3') [31], DGCR8 (5'-AUCCGUUGAUCUCGAGGAAdTdT-3', 5'-AACAUCGGACAAGAGUGUGAUdTdT-3', 5'-AU-CACACUCUUGUCCGAUGdTdT-3') and control(5'-UAGAACGUCUAGGUAUCCCdTdT-3').

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2 (5) 1 . 2 / - 1 . ( ) . 1 - 2 , 15% 1 -2 . 0.4 - . , - .

2 / - 2 2 3/ 5- 1 were used for assaying HR and NHEJ repair efficiencies, respec- . - ( ) 4 . used as a negative control. GFP-positive cells were identified and . - . , pCBASce, cells were treated with siRNAs specifically targeting 4 .

- ( ) 0.1% (50 - .4, 150 , 5 , 0.1% -40) - tion using a SonicDismembrator (Fisher Scientific) (1 min, with 10s-on and 20s-off cycles). Anti-HA affnity gel (Sigma, E6779), - (, 54) - - (, -20) -, - - , . 5 4 ° . - dogenous protein pull-down, specific antibodies were added to the 4 ° 3 . , (, 424) (, 32) 2 . , - . - - .

- (20 - , .4, 20% , 0.5% -40, 1 $_2$, 0.5 , 1 , 1) - - , , ( ). - - 2/ 2 2 (, 323 1), 1 (, 04-0 3), 3 (, 021 1), 4 (, 05-), - (, 14 01), - 51(- 2) (, - 34), - (, 12 2), - 11 (, 100-142), - (, 21 5), - Ku70 antibody (SantaCruz, sc-5309), mouse anti-β-tubulin anti- (, 52 3), - (, 10), - 1 (, 50003), -53 1 (, 300-2 2), - (- , 025), - - (- , 01 1) - - (, 044).

Immunofluorescence

2 , 4% 15 - 0.2% -100 10 . - 1 3 ° , 1 3 ° . ( ). 5 ( ). 5 . - ies were used for immunofluorescence: - 51 (- 2) (, - 34), - 11 (, 100-142), - (, 3423), -53 1 (, -22 0), - 0 (, 300- 3), - 1 1 (, 3 10), - - ( ), - ( ). - - 3 (, 21 1), - - (, 512), - - 3 (, 230), - - (, 25).

. , 1 10 2 / - - . - , 2 / - - 2 - - , - 4 , 1% 20 15 15 50 (, 1 50 , 20 5 used) purified sRNAs in DMEM (final concentration on cells was 0.25 /). 2 / - - - . 1 10% 3 ° , 5% $_2$, and processed for flow cytometric analysis of GFP.

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