#### SUPPLEMENTARY INFORMATION

### Recognition and processing of branched DNA substrates by Slx1-Slx4 nuclease

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### SUPPLEMENTARY FIGURES



Supplementary Figure S1. Overall structure of apo Slx1-Slx4<sup>CCD3</sup>. (A) structure of *Tt*-Slx1-Slx4<sup>CCD3</sup> (present study). Slx1 nuclease domain is shown in yellow with  $\beta$ -strands in orange. RING domain is shown in blue. Slx4 is shown in green. Zinc ions are shown as gray spheres. (B) *C. glabrata* Slx1-Slx4<sup>CCD3</sup> (PDB ID: 4XLG).



Supplementary Figure S2. Simulated annealing composite-omit maps for *Tt*-Slx1-Slx4<sup>CCD3</sup>–DNA structure. 2Fo-Fc map contoured at 1.1  $\sigma$  is shown for the DNA model (A) and selected DNA-binding residues (B).



**Supplementary Figure S3. Structure of the DNA.** (Left) Structure of the DNA from the *Tt*-Slx1-Slx4<sup>CCD3</sup>– DNA structure. The DNA is shown in cyan for canonical base pairs, in blue for single G-A mismatch and in gray for unpaired bases (Right) Schematic of base pairing in this DNA.

			10	20	30	40	50	60	70
			.	. <u>.</u>	<u>.</u>	<u></u>   <u>.</u>			
Tte	~~~~~~~~	~~~~ <b>MTV</b>	QCKPIPALYTV	YVLRSTV <mark>R</mark> ~~	HASLYIGSTPNPP	<mark>RR</mark> L <mark>K</mark> Q <mark>H</mark> NGLV	PGGAARTSRS	SLRPWEMVALV	SGFPSMVAA
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			10	20	30	40	50	60	70
-				•   • • • •   • ~ ′	*				
Cga	~~~~~~~~	· ~ ~ ~ ~ ~ ~ <u>ME</u>	EFQQIPDFYGC	YLLQSIS <mark>K</mark> ~~	RQSFYIGSTPNPV	RRLRQHNGSL	SRGGAYRTKRD	GTRPWEMVAIV	YGFPS <mark>R</mark> IAA
Cgl	~~~~~~~~~	·····MAT	VHSPIPALYTV	YILRSTVR~	HASFYVGSTPNPP	RRLSQHNGLV	RGGAVRTSRG	NLRPWEMIILV	SGFPSATAA
Vda	~~~~~~~~~	~~~~~ <b>MAV</b>	LSRPIPALYTV	YILRSTVR~	HASLYIGSTPNPP	RRLKQHNGLA	RGGAARTSRS	SLRPWEMIAIV	SGFPSMIAA
Cim	MYSEPDSMGHTE	PSYEPPSQEAV	~~KPIPAFYCA	YLLRSTVR~	HASLYIGSTPNPA	RRLAQHNGLI	KGGAKRTHKD	SLRPWEMVMLV	SGFMSRTAA
Teq	~~~~MPRSSRSS	PAPGNSRESA	~~KGIPPFYCV	ILLRSAVR~	HASLYIGSTPNPA	RRLAQHNGHI	KGGAHRTHRE	KLRPWEMVMIV	SGFTSRTAA
Ate	~~~~~~~	MUNVDSDQT	~~KPIPAYYCC	ILLRSTVN~I	RAGLYVGSTPNPP	RRLPQHNGLS	KGGAKKTATK	~NRPWEMVLLV	EGFMSRTAA
PUI	~~~~~~~	MIDGLKIDS	MDI CNEYCCI	LLRSKN	REAFILGSTPNPA	RRLGQHNGSS	NGGARRISMQ	GREWENTCIN	VCEDNKUCA
Sor			MARKEUREYCCI	TTTV2KKKY	SGAVIIGSIPDPP	RELEVINGET	OCCAWKTRN~	CRPWSISCLV	UCEDNKISA
SCI			MARKENFF ICC.	I LIQSINNA.	55K5L11G51FNF1	KKLKQHNGEI	QGGAWINI KI	GREWIVICLV	HGFFNRISA
	80	90	100		10 120	130	140	150	160
							.		
Tte	LKFEWALTNPHI	SVHIPSASRL	TVA~~~~~~	TOTKANGRPO	RPPRSLASVVANL	HLLLRVPSFA	RWPLRVHFFRR	DVFAAWEKWCA	AASER~~~LRP
				~ · ·					
	80	90	100		110	120	130	140	150
			••						
Cga	L <mark>Q</mark> F <mark>EH</mark> AWQ <mark>H</mark> GYÇ	TRYIKSQDRV	<b>VKT</b> ~~~~~~~	~~~~~	R <mark>K</mark> GG <mark>R</mark> SIHHKLAMI	TSLLKNEYFR	YMDLTLHFFNQ	KVEEIWKNDKE	NVSQTQESIDN
Cgl	LKFEWALNNPHI	SMHIPSAERL	VVSTQRNR~~~	~~~~ <b>NGRP</b> I	RPAKSLASVASSL	HLLLRVPSFA	RWPLCVQFFNR	DAFAAWEKWCA	GVSLGERGLRE
Vda	TREEWATENDUT	OT UT DOBOD T							
	DIVERMENTINEUT	STHIPSESKI	SRAAGVKK~~~~	~~~~NGHPI	RPRPGITSIMSNL	HLLLRVPSFE	RWPLKLHFFVK	TAHKAWNDSCA	AAEK~~~PVRK
Cim	LQFEWAWQHTPS	SRHADHEDES	SQPPVQI~~YPI	RSERRAKRS	KRPRPGITSIMSNL SRPRSSLKSILKSL	HLLLRVPSFEI HLLLRSPYFS	RWPLKLHFFVK VWPLEVHFFSA	TAHKAWNDSCA EIYRAWQGCCÇ	AAEK~~~PVRK LLDN~~~LIPD
Cim Teq	LQFEWAWQHTPS LQFEWAWQNTQF	SRHADHEDES SRHADHEDES SRHATGDEIE	SQPPVQI~~YPI TKVRICSKT~~~	NGHPI RSERRAKRS: -GKRLAKKS:	KRPRPGITSIMSNL SRPRSSLKSILKSL SNPREPMTSIMARL	HLLLRVPSFEI HLLLRSPYFS HVLLRSPYFS:	RWPLKLHFFVK VWPLEVHFFSA SWPLQVQFFNA	TAHKAWNDSCA EIYRAWQGCCÇ DIHRVWQGWVE	AAEK~~~PVRK LLDN~~~LIPD SAST~~~FVPE
Cim Teq Ate	LQFEWAWQHTPS LQFEWAWQNTQF LQFEWAWQNEDS	SCHIPSESRI SRHADHEDES SRHATGDEIE SRHMSKGEPGN	SRAAGVKK~~~~ SQPPVQI~~YPI TKVRICSKT~~~ TK~~~~~	NGHPI RSERRAKRS -GKRLAKKS RR(	KRPRPGITSIMSNL SRPRSSLKSILKSL SNPREPMTSIMARL QRPRRSLTANLEKL	HLLLRVPSFEI HLLLRSPYFS HVLLRSPYFS: HSLLQSPCFSI	RWPLKLHFFVK VWPLEVHFFSA SWPLQVQFFNA RWPLNIRIFAS	TAHKAWNDSCA EIYRAWQGCCQ DIHRVWQGWVE DVYQLWRVWCE	AAEK~~~PVRK LLDN~~~LIPD SAST~~~FVPE RANG~~~TIPE
Cim Teq Ate Ptr	LQFEWAMQHTPS LQFEWAWQNTQA LQFEWAWQHEDS LQFEWAWQNTHA	SCHIPSESSI SRHADHEDES SRHATGDEIE SRHMSKGEPGN ATRHIERDVRE	SKAAGVKK SQPPVQI~YPI TKVRICSKT TK ARKDELEKGRKI	NGHPI RSERRAKRS: GKRLAKKS: RR( NASP~~VKR:	KRPRPGITSIMSNL SRPRSSLKSILKSL SNPREPMTSIMARL QRPRRSLTANLEKL SRPPMSLEARLKNL	HLLLRVPSFEI HLLLRSPYFS HVLLRSPYFS HSLLQSPCFSI HHLLGVGSFSI	RWPLKLHFFVK VWPLEVHFFSA SWPLQVQFFNA RWPLNIRIFAS RWPLHVRFFAP	TAHKAWNDSCA EIYRAWQGCCQ DIHRVWQGWVE DVYQLWRVWCD DVFSQWEKHIS	LAAEK~~~PVRK OLLDN~~~LIPD SAST~~~FVPE ORANG~~~TIPE SKMNT~~~SLRK
Cim Teq Ate Ptr Spo	LQFEWAWQHTPS LQFEWAWQNTQA LQFEWAWQHEDS LQFEWAWQNTHA LKFEWNWQNLGJ	SSCHIPSESSI SSRHADHEDES ASRHATGDEIE SRHMSKGEPGN ATRHIERDVRE SSRYTKDCDFR	SRAAGVKK~~~~ SQPPVQI~~YPI TKVRICSKT~~~ TK~~~~ ARKDELEKGRKI S~~~~~	NGHPI RSERRAKRS: GKRLAKKS: RR( NASP~~VKR:	(RPRPGITSIMSNL SRPRSSLKSILKSL SNPREPMTSIMARL QRPRRSLTANLEKL SRPPMSLEARLKNL - KKQKTIMYCLKGL	HLLLRVPSFEI HLLLRSPYFS' HVLLRSPYFS: HSLLQSPCFSI HHLLGVGSFSI KHLVDSDTWRI	RWPLKLHFFVK VWPLEVHFFSA SWPLQVQFFNA RWPLNIRIFAS RWPLHVRFFAP RWPLNITFLNK	TAHKAWNDSCA EIYRAWQGCCQ DIHRVWQGWVE DVYQLWRVWCE DVFSQWEKHIS TAFSKWNQLGA	LAAEK~~~PVRK DLLDN~~~LIPD SAST~~FVPE DRANG~~~TIPE SKMNT~~~SLRK XTYGN~~~~~
Cim Teq Ate Ptr Spo Scr	LQFEWAWQHTPS LQFEWAWQNTQZ LQFEWAWQHEDS LQFEWAWQHEDS LQFEWAWQNTHZ LKFEWNWQNLGI LQFEWIWQHPNI	SSLHIPSESRI SSRHADHEDES ASRHATGDEIE SRHMSKGEPGN ATRHIERDVRE SSRYTKDCDFR SSRHTKDKEAK	SRAAGVKK SQPPVQI~YPI TKVRICSKT~~ TK~~~ ARKDELEKGRKI S~~~~ I	NGHPI RSERRAKRS GKRLAKKS NASP~~VKR	(RPRPGITSIMSNL SRPRSSLKSILKSL SNPREPMTSIMARL 2RPRRSLTANLEKL SRPPMSLEARLKNL - KKQKTIMYCLKGL - NKTPSLSNSLVAL	HLLLRVPSFEI HLLLRSPYFS HVLLRSPYFS HSLLQSPCFSI HHLLGVGSFSI KHLVDSDTWRI QQIVSCNGWNI	RWPLKLHFFVK VWPLEVHFFSA SWPLQVQFFNA RWPLNIRIFAS RWPLHVRFFAP RWPLNITFLNK RWPLEITFFSQ	TAHKAWNDSCA EIYRAWQGCCQ DIHRVWQGWVE DVYQLWRVWCE DVFSQWEKHIS TAFSKWNQLGA HAFEKWKAISA	LAAEK PVRK DLLDN LIPD SAST FVPE RANG TIPE SKMNT SLRK TYGN CGN
Cim Teq Ate Ptr Spo Scr	LQFEWANQATTQZ LQFEWANQATTQZ LQFEWANQATQZ LQFEWANQATAZ LKFEWNWQNLGI LQFEWIWQHPNI	SSHHPSESRI SSRHADHEDES ASRHATGDEIE GRHMSKGEPGN ATRHIERDVRE SSRYTKDCDFR SSRHTKDKEAK	SRAAGVKK SQPPVQI~YPI TKVRICSKT~~ TK~~ ARKDELEKGRKI S~~~~ I	RSERRAKRS GKRLAKKS NASPVKR	KRPRGITSIMSNL SRPRSSLKSILKSL SNPREPMTSIMARL SRPRRSLTANLEKL SRPPMSLEARLKNL - KKQKTIMYCLKGL - NKTPSLSNSLVAL	HLLLRVPSFEI HLLLRSPYFS HVLLRSPYFS: HSLLQSPCFSI HHLLGVGSFSI KHLVDSDTWRI QQIVSCNGWNI	RWPLKLHFFVK VWPLEVHFFSA SWPLQVQFFNA RWPLNIRIFAS RWPLHVRFFAP RWPLNITFLNK RWPLEITFFSQ	TAHKAWNDSCZ EIYRAWQGCCÇ DIHRVWQGWVE DVYQLWRVWCL DVYSQWEKHIS TAFSKWNQLGF HAFEKWKAISF	AAEK PVRK DLDN VIP SAST FVPE RANG SLRK KMNT SLRK TYGN GN
Cim Teq Ate Ptr Spo Scr	LQFEWAWQHTPF LQFEWAWQNTQF LQFEWAWQHEDS LQFEWAWQHEDS LQFEWAWQNTHA LKFEWNWQNLGI LQFEWIWQHPNI 170	SSRHADHEDES SSRHATGDEIE SRHMSKGEPGN ATRHIERDVRE SRYTKDCDFR SRHTKDKEAK 180	SKAAGVKK~~~ SQPPVQI~~YPI TKVRICSKT~~ TK ARKDELEKGRKI S~~~~~~~	RSERRAKRSS GKRLAKKSS NASP VKRS	RPRPGITSIMSNL SRPRSSLKSILKSL SNPREPMTSIMARL QRPRSLTANLEKL SRPPMSLEARLKNL - KKQKTIMYCLKGL - NKTPSLSNSLVAL	HLLLRVPSFEI HLLLRSPYFS: HVLLRSPYFS: HSLLQSPCFSI HHLLGVGSFSI KHLVDSDTWRI QQIVSCNGWNI	RWPLKLHFFVK VWPLEVHFFSA SWPLQVQFFNA RWPLNIRIFAS RWPLHVRFFAP RWPLNITFLNK RWPLEITFFSQ	TAHKAWNDSC2 EIYRAWQGCC2 DIHRVWQGWVE DVYQLWRVWCI DVFSQWEKHIS TAFSKWNQLGF HAFEKWKAISK	LAAEK PVRK LLDN LIPD SAST FVPE NRANG TIPE KKMNT SLRK XTYGN GN
Cim Teq Ate Ptr Spo Scr	LQFEWAWQHT9F LQFEWAWQHT0F LQFEWAWQHEDS LQFEWAWQHEDS LQFEWAWQHEDS LQFEWAWQHEDS LQFEWIWQHPN1 170	ISLHIPSESRI SRHADHEDES ASRHATGDEIE SRHMSKGEPGN ITRHIERDVRE SRYTKDCDFR SRHTKDKEAK 180 	SKAAGVK~~~YPI TKVRICSKT~~ ARKDELEKGRKI S~~~~~	Serrakrs: SERRAKRS: GKRLAKKS: NASP~~VKR:	RRPRGITSIMSNL SRPRSSLKSILKSL SNPREPMTSIMARL QRPRRSLTANLEKL SRPPMSLEARLKNL KKQKTIMYCLKGL	HLLLRVPSFEI HLLLRSPYFS: HVLLRSPYFS: HSLLQSPCFSI HHLLGVGSFSI KHLVDSDTWRI QQIVSCNGWNI	RWELKLHFFVK VWPLEVHFFSA SWELQVQFFNA RWPLNIRIFAS RWPLNIRIFAP RWPLNITFLNK RWPLEITFFSQ	TAHKAWNDSC2 EIYRAWQGCCQ DIHRVWQGWVE DVYQLWRVWCC DVFSQWEKHIS TAFSKWNQLGF HAFEKWKAISF	LALEK ~~~ PVRK PLLDN ~~~ LIPD SAST ~~~ FVPE RANG ~~~ TIPE KMNT ~~~ SLRK TYGN ~~~~~~ CGN ~~~~~~~~
Cim Teq Ate Ptr Spo Scr <b>Tte</b>	LQFEWAWQHTQF LQFEWAWQHTQF LQFEWAWQHEDS LQFEWAWQHEDS LQFEWAWQNLGJ LQFEWIWQHPNI 170 .    SLAVVTDFEGGS	SSRHADHEDES SSRHADHEDES SSRHATGDEIE SRHMSKGEPGN ITRHIERDVRE SSRYTKDCDFR SSRHTKDKEAK 180 	SKAAGVKK~~~YPI TKVRICSKT~~~ TKACLEKGRKI S~~~~~~~~~~~ I	NGHPI RSERRAKRS: -GKRLAKKS: NASPVKR:	RPRPGITSIMSNL SRPRSSLKSILKSL SNPREPMTSIMARL ORPRRSLTANLEKL SRPPMSLEARLKNL KKQKTIMYCLKGL	HLLLRVPSFEI HLLLRSPYFS HVLLRSPYFS: HSLLQSPCFSI HHLLGVGSFSI KHLVDSDTWRI QQIVSCNGWNI	RWELKLHFFVK VWPLEVHFFSA SWPLQVQFFNA RWPLNIRIFAS RWPLHVRFFAP RWPLNITFINK RWPLEITFFSQ	TAHKAWNDSCZ EIYRAWQGCCQ DIHRVWQGWVE DVYQLWRVWCI DVYSQWEKHIS TAFSKWNQIGH HAFEKWKAISK	LALEK ~~~ PVRK PLLDN ~~~ LYPC SAST ~~~ FVPE RANG ~~~ TIPE KMNT ~~~ SLRK TYGN ~~~~~~ CON~~~~~~~~
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Cim Teq Ate Ptr Spo Scr <b>Tte</b>	LQFEWAWQHTQF LQFEWAWQNTQF LQFEWAWQNTQF LQFEWAWQNTDF LQFEWAWQNTHF LKFEWNWQNLGI LQFEWIWQHPNI 170 	SSRHADHEDES SSRHATGDEIE SRHMSKGEPGN TTRHIERDVRE SSRYTKDCDFR SRHTKDKEAK 180 	SKAAGVKA~~~YPI SQPPVQI~~YPI TKVRICSKT~~~ ARKDELEKGRKI S~~~~~~	NERLAKS:	RPRPGITSIMSNL SRPRSSLKSILKSL SNPREPMTSIMARL QRPRSLTANLEKL SRPPMSLEARLKNL - KKQKTIMYCLKGL - NKTPSLSNSLVAL	HLLLRVPSFEI HLLLRSPYFS HVLLRSPYFS: HSLLQSPCFSI HHLLGVGSFSI KHLVDSDTWRI QQIVSCNGWNI	RWPLKLHFFVK VWPLEVHFFSA SWPLQVQFFNA RWPLNIRIFAS RWPLHVRFFAP RWPLNITFLNK RWPLEITFFSQ	TAHKAWNDSC2 EIYRAWQGCCQ DIHRVWQGWVE DVYQLWRVWCL DVFSQWEKHIS TAFSKWNQLGF HAFEKWKAISF	LALEK ~~~ PVRK LLDN~~ LIPD SAST~~~ FVPE SRANG~~~ TIPE SKMNT~~~ SLRK TYGN~~~~~~ GN~~~~~~~
Cim Teq Ate Ptr Spo Scr Tte	LQFEWAWQHTPF LQFEWAWQNTQF LQFEWAWQHEDS LQFEWAWQHEDS LQFEWAWQNTAF LKFEWNWQNLGI LQFEWIWQHPNI 170 	SSRHADHEDES SSRHADHEDES SSRHATGDEIE SRHMSKGEPGN ITRHIERDVRE SSRHTKDCDFR SSRHTKDKEAK 180 	SKAAGVK~~~YPI TKVRICSKT~~~ TK~~~~~~~ ARKDELEKGRKI S~~~~~~~	NERLAKKS	RPRPGITSIMSNL SRPRSSLKSILKSL SNPREPMTSIMARL QRPRSLTANLEKL SRPPMSLEARLKNL - KKQKTIMYCLKGL - NKTPSLSNSLVAL	HLLLRVPSFEI HLLLRSPYFS HVLLRSPYFS: HSLLQSPCFSI HHLLGVGSFSI KHLVDSDTWRI QQIVSCNGWNI	RWELKLHFFVK VWPLEVHFFSA SWPLQVQFFNA RWPLNIRIFAS RWPLHVRFFAP RWPLNITFLNK RWPLEITFFSQ	TAHKAWNDSC2 EIYRAWQGCCQ DIHRVWQGWVE DVYQLWRVWCI DVFSQWEKHIS TAFSKWNQLGF HAFEKWKAISF	LALEK ~~~ PVRK LLDN ~~~ LIPD SSAST ~~ FVPE DRANG ~~~ TIPE KMNT ~~~ SLRK KTYGN ~~~~ ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Cim Teq Ate Ptr Spo Scr <b>Tte</b>	LQFEWAWQHT9F LQFEWAWQHTQF LQFEWAWQHEDS LQFEWAWQHEDS LQFEWAWQHEDS LQFEWAWQHEDS LQFEWIWQHPNI 170 	SSRHADHEDES SSRHADHEDES SSRHATGDEIE SRHMSKGEPGN TTRHIERDVRE SRHTKDKEAK 180 	SKAAGVK~~~YPI SQPPVQI~~YPI TKVRICSKT~~~ ARKDELEKGRKI S~~~~~~~~	RSERRAKRS: GRRLAKKS: NASP~VKR:	RPRPGITSIMSNL SRPRSSLKSILKSL SNPREPMTSIMARL ORPRRSLTANLEKL SRPPMSLEARLKNL KKQKTIMYCLKGL	HLLLRVPSFEI HLLLRSPYFS HVLLRSPYFS: HSLLQSPCFSI HHLLGVGSFSI KHLVDSDTWRI QQIVSCNGWNI	RWELKLHFFVK VWPLEVHFFSA SWELQVQFFNA RWPLNIRIFAS RWPLNVRFFAP RWPLNITFLNK RWPLEITFFSQ	TAHKAWNDSC2 EIYRAWQGCCQ DIHRVWQGWVE DVYQLWRVWCI DVFSQWEKHIS TAFSKWNQIGF HAFEKWKAISF	LALEK ~~~ PVRK PLLDN ~~~ LIPD SAST ~~ FVPE RANG ~~~ TIPE KMNT ~~~ SLRK TYGN ~~~~~~ CGN ~~~~~~~~
Cim Teq Ate Ptr Spo Scr <b>Tte</b> Cga Cg1 Vda	LQFEWAWQHT9F LQFEWAWQHTQF LQFEWAWQHEDS LQFEW	SSRHADHEDES SSRHADHEDES SSRHATGDEIE SRHMSKGEPGN TRHIERDVRE SRYTKDCDFR SRHTKDKEAK 180 	SKAAGVKA~~~YPI TKVRICSKT~~~ TK~~~~~~~ ARKDELEKGRKI S~~~~~~~~~~	NGREIAKKS: GRRLAKKS: NASP~VKR:	RPRPGITSIMSNL SRPRSSLKSILKSL SNPREPMTSIMARL QRPRRSLTANLEKL SRPPMSLEARLKNL KKQKTIMYCLKGL -NKTPSLSNSLVAL	HLLLRVPSFEI HLLLRSPYFS HVLLRSPYFS HSLLQSPCFSI HHLLGVGSFSI KHLVDSDTWRI QQIVSCNGWNI	RWELKLHFFVK VWPLEVHFFSA SWPLQVQFFNA RWPLNIRIFAS RWPLHVRFFAP RWPLNITFLNK RWPLEITFFSQ	TAHKAWNDSC2 EIYRAWQGCCQ DIHRVWQGWVE DVYQLWRVWCI DVFSQWEKHIS TAFSKWNQIGF HAFEKWKAISF	LALEK ~~~ PVRK LLDN ~~~ LVPD SAST ~~~ FVPE RANG ~~~ TIPE KMNT ~~~ SLRK TYGN ~~~~ ~~~ CON ~~~~~~~~
Cim Teq Ate Ptr Spo Scr <b>Tte</b> Cga Cg1 Vda Cim	LQFEWAWQHT9F LQFEWAWQNT0F LQFEWAWQNT0F LQFEWAWQNT0F LQFEWAWQNT0F LQFEWIWQHDNI SLAVVTDFEGGS 170 	SSHADHEDES SSRHATGDEIE SRHMSKGEPGN ATRHIERDVRE SSRYTKDCDFR SRHTKDCKEAK 180 	SKAAGVKK~~~~ SQPPVQI~~YPI TKVRICSKT~~~ ARKDELEKGRKI S~~~~~~~~~~	GRILAKSS GRILAKSS NASP~VKRS	RPRPGITSIMSNL SRPRSSLKSILKSL SNPREPMTSIMARL ORPRRSLTANLEKL SRPPMSLEARLKNL KKQKTIMYCLKGL •NKTPSLSNSLVAL	HLLLRVPSFEI HLLLRSPYFS HVLLRSPYFS: HSLLQSPCFSI HHLLGVGSFSI KHLVDSDTWRI QQIVSCNGWNI	RWELKLHFFVK VWPLEVHFFSA SWPLQVQFFNA RWPLNIRIFAS RWPLHVRFFAP RWPLNITFINK RWPLEITFFSQ	TAHKAWNDSC2 EIYRAWQGCCQ DIHRVWQGWVE DVYQLWRVWCI DVFSQWEKHIS TAFSKWNQIGF HAFEKWKAISF	LALEK ~~~ PVRK LLDN ~~~ LVRK SAST ~~~ FVPE SRANG ~~~ TIPE SKMNT ~~~ SLRK TYGN ~~~~~~ CON ~~~~~~~~
Cim Teq Ate Ptr Spo Scr <b>Tte</b> Cga Cg1 Vda Cim Teq	LQFEWAWQHTOF LQFEWAWQNTOF LQFEWAWQNTOF LQFEWAWQNTOF LQFEWAWQNTHF LKFEWNWQNLGI LQFEWIWQHPNI 170 	SSRHADHEDES SSRHATGDEIE SRHMSKGEPGN TTRHIERDVRE SSRYTKDCDFR SRHTKDCEAK 180 	SKAAGVKA~~~YPI SQPPVQI~~YPI TKVRICSKT~~~ ARKDELEKGRKI S~~~~~~	NERLAKSS	RPRPGITSIMSNL SRPRSSLKSILKSL SNPREPMTSIMARL QRPRSLTANLEKL SRPPMSLEARLKNL - KKQKTIMYCLKGL - NKTPSLSNSLVAL	HLLLRVPSFEI HLLLRSPYFS HVLLRSPYFS: HSLLQSPCFSI HHLLGVGSFSI KHLVDSDTWRI QQIVSCNGWNI	RWELKLHFFVK VWPLEVHFFSA SWPLQVQFFNA RWPLNIRIFAS RWPLHVRFFAP RWPLNITFLNK RWPLEITFFSQ	TAHKAWNDSC2 EIYRAWQGCCQ DIHRVWQGWVE DVYQLWRVWCI DVYSQWEKHIS TAFSKWNQLGF HAFEKWKAISF	LALEK ~~~ PVRK LLDN ~~~ LIPD SSAT ~~ FVPE PRANG ~~~ TIPE KMNT ~~~ SLRK (TYGN ~~~~~~~ (GN ~~~~~~~~~
Cim Teq Ptr Spo Scr <b>Tte</b> Cga Cg1 Vda Cim Teq Ate	LQFEWAWQHT9F LQFEWAWQNT9F LQFEWAWQNT9F LQFEWAWQNT4F LKFEWNWQNLGI LQFEWIWQHPNI 170 	SSRHADHEDES SSRHADHEDES SSRHATGDEIE SRHMSKGEPGN ITRHIERDVRE SSRYTKDCDFR SSRHTKDKEAK 180 	SKAAGVKA~~~YPI TKVRICSKT~~~ TK~~~~~~~ ARKDELEKGRKI S~~~~~~~~	RESERAKRS: CERLLAKS: NASP~VKR:	RPRPGITSIMSNL SRPRSSLKSILKSL SNPREPMTSIMARL QRPRSLTANLEKL SRPPMSLEARLKNL KKQKTIMYCLKGL	HLLLRVPSFEI HLLLRSPYFS HVLLRSPYFS HSLLQSPCFSI HHLLGVGSFSI KHLVDSDTWRI QQIVSCNGWNI	RWELKLHFFVK VWPLEVHFFSA SWPLQVQFFNA RWPLNIRIFAS RWPLHVRFFAP RWPLNITFLNK RWPLEITFFSQ	TAHKAWNDSC2 EIYRAWQGCCQ DIHRVWQGWVE DVYQLWRVWCI DVFSQWEKHIS TAFSKWNQLGF HAFEKWKAISF	LALEK ~~~ PVRK LLDN ~~~ LIPD SAST ~~ FVPE RANG ~~~ TIPE KMNT ~~~ SLRK CTYGN ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Cim Teq Ate Ptr Spo Scr <b>Tte</b> Cga Cgl Vda Cim Teq Ate Ptr	LQFEWAWQHTPF LQFEWAWQNTQF LQFEWAWQNTAF LQFEWAWQNTAF LKFEWNWQNLGI LQFEWIWQHPNI 170 	SSRHADHEDES SSRHADHEDES SSRHATGDEIE SRHMSKGEPGN ITRHIERDVRE SSRTKDCDFR SSRTKDCDFR SSRTKDCDFR SEGLAGA 180 	SKAAGVKA~~~YPI SQPPVQI~~YPI TKVRICSKT~~~ ARKDELEKGRKI S~~~~~~~~~	RSERRAKRS	RPRPGITSIMSNL SRPRSSLKSILKSL SNPREPMTSIMARL QRPRRSLTANLEKL SRPPMSLEARLKNL KKQKTIMYCLKGL	HLLLRVPSFEI HLLLRSPYFS HVLLRSPYFS: HSLLQSPCFSI HHLLGVGSFSI KHLVDSDTWRI QQIVSCNGWNI	RWELKLHFFVK VWPLEVHFFSA SWPLQVQFFNA RWPLNIRIFAS RWPLHVRFFAP RWPLNITFLNK RWPLEITFFSQ	TAHKAWNDSC2 EIYRAWQGCCQ DIHRVWQGWVE DVYQLWRVWCI DVFSQWEKHIS TAFSKWNQIGF HAFEKWKAISF	LALEK ~~~ PVRK LLDN ~~~ LIPD SAST ~~ FVPE RANG ~~~ TIPE KMNT ~~~ SLRK TYGN ~~~~~~ CGN ~~~~~~~~
Cim Teq Ate Ptr Spo Scr <b>Tte</b> Cga Cg1 Vda Cdim Teq Ate Ptr Spo	LQFEWAWQHT9F LQFEWAWQHTQF LQFEWAWQHEDS LQFEWAWQHEDS LQFEWAWQHEDS LQFEWAWQHEDS LQFEWAWQHEDS LQFEWAWQHEDS LQFEWAWQHEDS 170 	SSRHADHEDES SSRHADHEDES SSRHATGDEIE SRHMSKGEPGN TTRHIERDVRE SRHTKDCDFR SRHTKDKEAK 180 	SKAAGVKA~~~YPI SQPPVQI~~YPI TKVRICSKT~~~ TK~~~~~~~~~ ARKDELEKGRKI S~~~~~~~~~~	RSERRAKRS: CGRLLAKS: NASP~VKR:	RPRPGITSIMSNL SRPRSSLKSILKSL SNPREPMTSIMARL ORPRRSLTANLEKL SRPPMSLEARLKNL KKQKTIMYCLKGL -NKTPSLSNSLVAL	HLLLRVPSFEI HLLLRSPYFS HVLLRSPYFS HSLLQSPCFSI HHLLGVGSFSI KHLVDSDTWRI QQIVSCNGWNI	RWELKLHFFVK VWPLEVHFFSA SWPLQVQFFNA RWPLNIRIFAS RWPLHVRFFAP RWPLNITFLNK RWPLEITFFSQ	TAHKAWNDSC2 EIYRAWQGCCQ DIHRVWQGWVE DVYQLWRVWCI DVFSQWEKHIS TAFSKWNQIGF HAFEKWKAISF	LALEK ~~~ PVRK LLDN ~~~ IIPD SAST ~~ FVPE RANG ~~~ TIPE KMNT ~~~ SLRK TYGN ~~~~ SLRK CYGN ~~~~~~~

Supplementary Figure S4. Multiple sequence alignment of nuclease domains of fungal Slx1 proteins. Secondary structure elements are shown for *Tte*-Slx1 (arrows for  $\beta$ -stands and cylinders for  $\alpha$ -helices). Residues that are involved in DNA binding at the new interface are highlighted in purple. Active-site residues are shown in yellow. Predicted site I and II residues are shown in cyan. Tte, *Thelavia terrestris*; Cga, *Candida glabrata*; Pgt, *Puccinia graminis tritici*; Cgl, *Chaetomium globosum*; Vda, *Verticillium dahlia*; Cim, *Coccidioides immitis*; Teq, *Trichophyton equinum*; Ate, *Aspergillus terreus*; Ptr, *Pyrenophora triticirepentis*; Spo, *Schizosaccharomyces pombe*; Scr, *Schizosaccharomyces cryophilus*.



Supplementary Figure S5. Representative gels that were used to quantify the activity of various mutants of *Cg*-Slx1-Slx4<sup>CCD</sup> on 5'-flap substrate. (A) Activity test of site III mutants. (B) Activity test of site II mutants. (C) Activity tests of site I mutants together with a mutant in which site II and site III were mutated. Upper gels in each panel present the activity of the Cy5-labeled strand. Lower gels in each panel present the activity of the Cy5-labeled strand. Lower gels in each panel products of the reaction (P) are labeled on the right side of the panels.



**Supplementary Figure S6. Secondary structure content and structural integrity of** *Cg***-Slx1-Slx4**<sup>CCD</sup> **variants.** Fourier-Transform Infrared (FT-IR) spectrum of *Cg*-Slx1-Slx4<sup>CCD</sup> and various mutants (Supplementary Table S3).

Oligon	ucleotides used to generate substrates for activity test and binding studies.
X0-1	ACGCTGCCGAATTCTACCAGTGCCTTGCTAGGACATCTTTGCCCACCTGCAGGTTCACCC
X0-2	GGGTGAACCTGCAGGTGGGCAAAGATGTCCATCTGTTGTAATCGTCAAGCTTTATGCCGT
X0-3	ACGGCATAAAGCTTGACGATTACAACAGATCATGGAGCTGTCTAGAGGATCCGACTATCG
X0-4	CGATAGTCGGATCCTCTAGACAGCTCCATGTAGCAAGGCACTGGTAGAATTCGGCAGCGT
X0-1 <sup>f</sup>	(Fluorescein)ACGCTGCCGAATTCTACCAGTGCCTTGCTAGGACATCTTTGCCCACCTGCAGGTTCACCC
X0-4 <sup>c</sup>	CGATAGTCGGATCCTCTAGACAGCTCCATGTAGCAAGGCACTGGTAGAATTCGGCAGCGT (Cy5)
X0-1 <sup>fc</sup>	(Fluorescein)ACGCTGCCGAATTCTACCAGTGCCTTGCTAGGACATCTTTGCCCACCTGCAGGTTCACCC (Cy5)
X0-2.30	GGGTGAACCTGCAGGTGGGCAAAGATGTCC
X0-3.30	CATGGAGCTGTCTAGAGGATCCGACTATCG
X0-4.30	TAGCAAGGCACTGGTAGAATTCGGCAGCGT
Oligonucleo	tides used for mapping cleavage sites
X0-1.28 <sup>f</sup>	(Fluorescein)ACGCTGCCGAATTCTACCAGTGCCTTGC
X0-1.29 <sup>f</sup>	(Fluorescein)ACGCTGCCGAATTCTACCAGTGCCTTGCT
X0-1.31 <sup>f</sup>	(Fluorescein)ACGCTGCCGAATTCTACCAGTGCCTTGCTAG
X0-1.32 <sup>f</sup>	(Fluorescein)ACGCTGCCGAATTCTACCAGTGCCTTGCTAGG
X0-4.28 <sup>c</sup>	GCAAGGCACTGGTAGAATTCGGCAGCGT(Cy5)
X0-4.29 <sup>c</sup>	AGCAAGGCACTGGTAGAATTCGGCAGCGT(Cy5)
Oligonucleo	tides used for crystallization
114a	CAATCGGCAATGACCTTTGGTCATTCAGCAGAT
114b	ATCTGCTGAATCTGGTTTCCAGATTGCCGATTG

# Supplementary Table S1. Sequences of oligonucleotides (written 5' to 3').

# Supplementary Table S2. Oligonucleotides that were used to generate DNA substrates.

Substrates	Unlabeled	Labeled		
Substrates used in crys	tallization			
Holliday junction	114a, 114b			
Splayed-arm	114a			
Substrates used in activ	vity test/fluorescence anisotropy			
Holliday junction	X0-1, X0-2, X0-3, X0-4	Fluorescent: X0-1 <sup>f</sup> , X0-2, X0-3, X0-4 <sup>c</sup>		
Replication fork	X0-1, X0-2.30, X0-3.30, X0-4	Fluorescent: X0-1 <sup>f</sup> , X0-2.30, X0-3.30, X0-4 <sup>c</sup>		
5'-flap	X0-1, X0-2.30, X0-4	Fluorescent: X0-1 <sup>f</sup> , X0-2.30, X0-4 <sup>c</sup>		
3'-flap	X0-1, X0-3.30, X0-4	Fluorescent: X0-1 <sup>f</sup> , X0-3.30, X0-4 <sup>c</sup>		
Splayed-arm	X0-1, X0-4	Fluorescent: X0-1 <sup>f</sup> , X0-4 <sup>c</sup>		
Nicked DNA	X0-1, X0-2.30, X0-4.30	Fluorescent: X0-1 <sup>fc</sup> , X0-2.30, X0-4.30		

<sup>f</sup> Fluorescein-labeled oligonucleotides. <sup>c</sup> Cy5-labeled oligonucleotides. <sup>fc</sup> Oligonucleotide labeled with

Fluorescein at 5' end and Cy5 at 3' end.

	Mutant name			
	Mutations in Site I			
1	R35A	Cg-Slx1 <sup>R35A</sup> -Slx4 <sup>CCD</sup>		
2	R38A	Cg-Slx1 <sup>R38A</sup> -Slx4 <sup>CCD</sup>		
	Mutations in Site II			
1	H80A	Cg-Slx1 <sup>H80A</sup> -Slx4 <sup>CCD</sup>		
2	H84A	Cg-Slx1 <sup>H84A</sup> -Slx4 <sup>CCD</sup>		
3	Site II	Cg-Slx1 <sup>H80A/H84A</sup> -Slx4 <sup>CCD</sup>		
	Mutations in Site III			
1	K21A	Cg-Slx1 <sup>K21A</sup> -Slx4 <sup>CCD</sup>		
2	R22A	Cg-Slx1 <sup>R22A</sup> -Slx4 <sup>CCD</sup>		
3	R101A	Cg-Slx1 <sup>R101A</sup> -Slx4 <sup>CCD</sup>		
4	K102A	Cg-Slx1 <sup>K102A</sup> -Slx4 <sup>CCD</sup>		
5	R105A	Cg-Slx1 <sup>R105A</sup> -Slx4 <sup>CCD</sup>		
	Mutations in Site II and Site III			
1	Site I + II	Cg-SIx1 <sup>H80A/H84A/K21A/R22A/R101A/K102A</sup> -SIx4 <sup>CCD</sup>		
	Others			
1	E79Q	Cg-Slx1 <sup>E79Q</sup> -Slx4 <sup>CCD</sup>		
2	Wild type	Cg-Slx1-Slx4 <sup>CCD</sup>		

Supplementary Table S3. *Cg*-Slx1-Slx4<sup>CCD</sup> mutants that were used in the study.