# Supplementary Table 1: Quantitation of Fig. 3A

## **Rai1 Exonuclease Activities**

			% RNA		% RNA		% RNA		% RNA
	Time (min)	m <sup>7</sup> GpppRNA	remaining	GpppRNA	remaining	pppRNA	remaining	pRNA	remaining
AgRai1	0	68348	100%	77240	100%	82903	100%	78637	100%
	20	23972	35%	34933	45%	35682	43%	34672	44%
	40	11910	17%	20741	27%	19292	23%	18451	23%
CaRai1	0	60647	100%	69637	100%	74044	100%	71644	100%
	20	62468	103%	69849	100%	72002	97%	68659	96%
	40	56275	93%	67564	97%	73880	100%	68944	96%
CgRai1	0	63809	100%	69298	100%	74902	100%	68120	100%
	20	63034	99%	64899	94%	71431	95%	67702	99%
	40	61359	96%	64682	93%	70740	94%	66891	98%
LtRai1	0	63738	100%	66623	100%	71012	100%	68254	100%
	20	50469	79%	57225	86%	61844	87%	58470	86%
	40	42093	66%	56179	84%	52841	74%	56177	82%
ScRai1	0	69709	100%	72589	100%	73853	100%	72406	100%
	20	70683	101%	75603	104%	74429	101%	69762	96%
	40	69482	100%	74206	102%	70063	95%	68462	95%
SpRai1	0	72493	100%	72941	100%	71030	100%	71240	100%
	20	72992	101%	71838	98%	67624	95%	71592	100%
	40	66817	92%	70880	97%	66296	93%	70131	98%
SsRai1	0	71487	100%	74401	100%	68885	100%	68795	100%
	20	51645	72%	57196	77%	49532	72%	43036	63%
	40	38077	53%	51153	69%	47862	69%	39374	57%
VpRai1	0	65864	100%	78180	100%	71742	100%	71122	100%
	20	71732	109%	74808	96%	71120	99%	68907	97%
	40	67409	102%	71853	92%	71529	100%	73001	103%

# Supplementary Table 2. Quantitation of Fig. 3C

## Rai1 Exonuclease Activities

		% RNA		% RNA		% RNA		% RNA
Time (min)	m <sup>7</sup> GpppRNA	remaining	GpppRNA	remaining	pppRNA	remaining	pRNA	remaining
0	122962	100%	88111	100%	102052	100%	95578	100%
20	72184	59%	67189	76%	72171	71%	82030	86%
40	35363	29%	37606	43%	38607	38%	19502	20%
0	101183	100%	92549	100%	93272	100%	95434	100%
20	102241	101%	87880	95%	88209	95%	87016	91%
40	87421	86%	82528	89%	92698	99%	85974	90%
	Time (min) 0 20 40 0 20 40	Time (min)m²GpppRNA0122962207218440353630101183201022414087421	% RNA     Time (min)   m <sup>7</sup> GpppRNA   remaining     0   122962   100%     20   72184   59%     40   35363   29%     0   101183   100%     20   102241   101%     40   87421   86%	% RNA     Time (min)   m <sup>7</sup> GpppRNA   remaining   GpppRNA     0   122962   100%   88111     20   72184   59%   67189     40   35363   29%   37606     0   101183   100%   92549     20   102241   101%   87880     40   87421   86%   82528	% RNA   % RNA     Time (min)   m <sup>7</sup> GpppRNA   remaining   GpppRNA   remaining     0   122962   100%   88111   100%     20   72184   59%   67189   76%     40   35363   29%   37606   43%     0   101183   100%   92549   100%     20   102241   101%   87880   95%     40   87421   86%   82528   89%	% RNA   % RNA     Time (min)   m <sup>7</sup> GpppRNA   remaining   GpppRNA   remaining   pppRNA     0   122962   100%   88111   100%   102052     20   72184   59%   67189   76%   72171     40   35363   29%   37606   43%   38607     0   101183   100%   92549   100%   93272     20   102241   101%   87880   95%   88209     40   87421   86%   82528   89%   92698	% RNA   % RNA   % RNA     Time (min)   m <sup>7</sup> GpppRNA   remaining   GpppRNA   remaining   pppRNA   remaining     0   122962   100%   88111   100%   102052   100%     20   72184   59%   67189   76%   72171   71%     40   35363   29%   37606   43%   38607   38%     0   101183   100%   92549   100%   93272   100%     20   102241   101%   87880   95%   88209   95%     40   87421   86%   82528   89%   92698   99%	% RNA   % RNA   % RNA   % RNA     Time (min)   m <sup>7</sup> GpppRNA   remaining   GpppRNA   remaining   pppRNA   remaining   pRNA     0   122962   100%   88111   100%   102052   100%   95578     20   72184   59%   67189   76%   72171   71%   82030     40   35363   29%   37606   43%   38607   38%   19502     0   101183   100%   92549   100%   93272   100%   95434     20   102241   101%   87880   95%   88209   95%   87016     40   87421   86%   82528   89%   92698   99%   85974

# Supplementary Table 3: Quantitation of Fig. 6C

### **Rai1 and mutants Exonuclease Activities**

			% RNA		% RNA		% RNA		% RNA
	Time (min)	m <sup>7</sup> GpppRNA	remaining	pppRNA	remaining	pRNA	remaining	OH-RNA	remaining
AgRai1	0	103507	100%	92192	100%	88274	100%	88271	100%
-	20	60724	59%	63127	68%	63799	72%	84584	96%
	40	52946	51%	59902	65%	57515	65%	81888	93%
AgRai1 K198I	0	86674	100%	82324	100%	77426	100%	82781	100%
	20	64381	74%	72817	88%	54253	70%	82327	99%
	40	41985	48%	66810	81%	40939	53%	83528	101%
AgRai1 E154A	0	84053	100%	78259	100%	82522	100%	84525	100%
	20	61048	73%	73217	94%	60193	73%	86228	102%
	40	55432	66%	68091	87%	57294	69%	86554	102%
AgRai1 Y159E	0	87361	100%	77335	100%	80672	100%	85425	100%
	20	85170	97%	75597	98%	80936	100%	85156	100%
	40	90459	104%	73949	96%	81561	101%	89198	104%
AgRai1	0	88925	100%	76338	100%	85540	100%	76028	100%
W154A/Y159E	20	81965	92%	79924	105%	85083	99%	77384	102%
	40	69236	78%	78811	103%	79157	93%	75545	99%
AgRai1 K110T	0	81278	100%	80404	100%	79451	100%	82541	100%
	20	69641	86%	71844	89%	69470	87%	79103	96%
	40	66005	81%	68281	85%	61978	78%	80435	97%
AgRai1	0	83542	100%	76464	100%	74862	100%	76536	100%
E154A/Y159E	20	85104	102%	74044	97%	80142	107%	76864	100%
K110T	40	85463	102%	79713	104%	80732	108%	77294	101%
CaRai1	0	83938	100%	86034	100%	92708	100%	92021	100%
	20	79772	95%	83164	97%	89668	97%	93115	101%
	40	78265	93%	81200	94%	87851	95%	88019	96%
CaRai1 E104G	0	109712	100%	97499	100%	79254	100%	97800	100%
	20	69677	64%	46699	48%	57724	73%	63986	65%
	40	68950	63%	34576	35%	53184	67%	63392	65%
CaRai1 E104K	0	80228	100%	84780	100%	91567	100%	85761	100%
	20	77300	96%	69028	81%	90382	99%	84840	99%
	40	68300	85%	68953	81%	85595	93%	79171	92%

AgRail 1 AgRail 127 Agrail 1	
s.s. AgRail 52 D.IEKHIDISAGARKFQDEQAEAEDDTGSÜHGLQTIMEYERRKSKKVNADIIAFRGQVKRLTHCAFGG CaRail 51 T.IDTGIDLQGGYSKFKKIPDEQNLADFNSLIKAIIKYETSEGKKISSDIITFREIMTKILSLPYNL SSRail 54 K.IDGSIDLQAGYAKFKKIPEEKNMSDMKCLLTALTKYEQEHNN.GEKVNVDIITYRGLMTKLLALPYNL SpRail 51 F.SDLMTGFPNRFHPPKSDPDPISIVKDVLMTKGIQNNSSFLTWRGLITKIMCAPLOPH SCRail 52 E.LDRKLDISGFQKFKDYYKDFE.DRCSLRGLLETIESSERHKGKKINADIITFRGIARKLISCAFDSI UPRAIL 52 E.LDNNLDLCGGIKKFRECFGNPDVDACTLSGLLKTIQAYEERKNKKVPADIITFRGINRKLISAAFDSI LTRAIL 52 E.LENNLDLCSGIKKFRECFGNPDVDACTLSGLLKTIQAYEERKNKKVPADIITFRGINRKLISAAFDSI CGRail 52 E.LENNLDLCSGIKKFRECFGNPDVDACTLSGLLKTIQAYEERKNKKVPADIITFRGINRKLISAAFDSI CGRail 52 F.JEHGTDLSAGASKFNDVAKDFK.DRCSLKGLLDTLMEYEKKKNKKVPADIITFRGINRKLISAAFDSI MMDX0 76 NGPGPDFDLRDGYPDRYQPRDEEVQERLDHLLRWVLEHRNQLEGGPGWLAGATVTWRGHLTKLLTPYER. S.S. B6 B7 AGD B8 AGRAIL 127 YVMSFDGOLFIRAARKKLEFPTSPRESWAYLAY'SGYKFERMALLDRPVAETPREVLESRK	YYYLPDA YYYLPDS YYYLPDS YYYLPDS YYYLPDA YYYLPDA YYYLPDA YYYLPDT YYYLPDT YYYLPDT YYYLPDT
AgRail 52 D.IEKHIDISAGARKFQDEQAEAEDDTGSIHGLUTIMEYERRKSKVVADIIAFRGVKRLTHCAFGG   Carail 51 T.IDTGIDLQGGYSKFKKIPDEQNLADFNSLLKALIKYETSEGKKISSDIIFREINTKILSLPYNL   SsRail 54 K.IDGSIDLQAGYAKFKKIPDEQNLADFNSLLKALIKYETSEGKKISSDIIFREINTKILSLPYNL   sprail 51 F.SDLNTGFPNRPHPPKSDPDPISIVKDVLMTKGEKVNVDIITYRGLMTKLLALPYNL   scrail 52 E.LDRKLDLSSGFQKFKDYKDFE.DRCSLRGLLETIESSERHKGKKINADIITFRGINTKLLALPYNL   scrail 52 E.LDRKLDLSSGFQKFKDYKDFE.DRCSLRGLLETIESSERHKGKKINADIITFRGINTKLLSCAPLDPI   vprail 52 E.LDRKLDLSSGFQKFKDYKDFE.DRCSLRGLLETIESSERHKGKKINADIITFRGINKLISCAFDSI   vprail 52 E.LDRKLDLSGIKFFKDFYKDFC.DPGTLKGLLKTIQAYEEKKNKKVPADITFRGINKLISAAFDSI   vgrail 52 E.LERNLDLCSGIKKFFKDFK.DFK.DRCSLKGLLDTLMEYEKKKNKKVPADITFRGINKLISAAFDSI   vgrail 52 E.LERNLDLSGIKFFKDVAKDFK.DRCSLKGLLDTLMEYEKKKNKVPADITFRGINKLISAAFDSI   vgrail 52 E.LERNLDLSGIKFFKDVAKDFK.DRCSLKGLLDTLMEYEKKKNKVSNVDIITFRGINKLISAAFDSI   mdxo 76 NGPGPDFDLRDGYPDRYQPRDEEVQERLDHLLRWVLEHRNQLEGGPGWLAGATVTWRGHLTKLLTTPYER.   MmDXO 76 NGPGPDFDLRDGYPDRYQPRDEEVQERLDHLLRWVLEHRNQLEGGPGWLAGATVTWRGHLTKLLDFFEVLESKC   S.S. 36 37   GCD 38	-
s.s. B6 B7 AD B8 AgRail 127 YVMSFDGOLFIRAARKKLEFPTSPRES	HATDVDM TDPIDL NDPVDL NHWETYL SFNTVDL KFNQVNL KYNRVDL SKYNRVDL QEGWQL
AgRail 127 YVMSFDCOLFIRAARKKLEFPTSPRES	
SSRail 128 NVLAYD GOLFINSDEEIELARRKEEDEHKQQSMTPEKYDHMKRCEFSGYKFEAIATLPKPWADCSRQQIDKG   SpRail 116 VMDPTSGIIMMEERTRSETSYANQDRMCYWGYKFEAISTLPEIWDACSRQQIDKG   ScRail 127 RIVSFNGOLFIKEVPEAVNAAKASSATEAGRNINQDLNVFTGYKFETLATLSNPLQYTPREVIEKR   vprail 127 RIVLFDGOIFMKEVPEAVNAAKASSATEAGRNINQDLNVFTGYKFETLATLSNPLQYTPREVIEKR   uprail 127 RIVLFDGOIFMKEVPEAVNAAKASSAT	K Q V R NG K K V V N NY S K K M V N NY D N Q D V V P D C K R I V S HG S K K I V S HG K K I C C NG R K I L N NG S G E V N T N
s.s. β9 β10 β11 β12	αF
AgRail 195 PQYKTVVRTGVGEHKLVLGAEVDGIPDER.EPTGDNLKHYVELKVAKKVQTLKI   CaRail 203 EQYLSVIRTGIGNVKLVLAGEIDCCWDVLPDEQKKKLNHYVELKTSRIJENNSG.   SsRail 208 EQYLSVIRTGIGAVKLVLAGEIDCCWDVLPDEQKKKLNHYVELKTSRIJENNSG.   SsRail 208 EQYLSVIRTGIGAKMLLAGEVDCVWDVLPDEQKKKLNHYVELKTSRIJENNSG.   SsRail 208 EQYLSVIKTGIGEAKMLLAGEVDCVWDVLPDEQKKKLSHYMELKTSRIJENNSG.   SsRail 109 EQYCSIVKINIGKSKLILAGEVDCVWDVLPEQKKVLSHYMELKTSRKYPLEN   ScRail 201 DEYISVVRTGVGNCKLLIGAEVDCIPDFK.ENGRDNLKHYAELKCTCQVANISI   VPRail 192 DEYISVVRTGVGNCKLLIGAEVDCIPDFK.EEDKDNLKHYVELTTNAVLTPSI   Ltrail 195 DQYIIVVVRSGVGHCKLVLGAEVDCIPDFT.EDSSDNLKHYVELTTNAVLTPSI   CgRail 206 DEYITVVRTGVGCKCKUVLGAEIDCIFDFK.EEGKDNLKHYELKCSSQISTTNI   MmDXO 214 VAYCSVLRSRLGNHPLESGEVDCLNPQAPCTQPPS	OATNFE.Q VVSFE.Q VVNFE.K .YGMR.K OTHKFE.R OARKFE.R CARAFE.R OVRRFE.K WRSFYRH
s.s. $\alpha F$ $\beta 13$ $\beta 14$ $\alpha G$ $\alpha H$	
AgRail 254 KLFSVWLOCFLVGINRVIIGFRDEKFVLKSVEEFSTSEIPLLLKSTGLRNACVDAIKWYGALTKWI CaRail   263 KLFKAWGOCFLWGVTKIIYGFRDNNLILKNVELFNTEEIPILIKNNPLTNAATEKKINCTMALKWYGAUVDWI SSRail 268 KLFKTWACCFLWGVTKIIYGFRDNNLILKNVELFNTEEIPILIKNNPLTNAATEKKINCTMALKWYGAUVDWI SSRail 268 KLLKYMACSFLGIGRIIIGFRDNSTINVE   SSRail 266 KLLKYMACSFLUGIGRIIIGFRDDNGILLEMKELFTHQIPKMLRPYFKPNDWTPNRLLVVLHALEWI VPRail 256 KLLKYMACSFLUGIGRIIIGFRDNNFILSVEEFSTEEVVLLKNNNPQVGSACLEAIKWYGULTEWI VIRAIL   251 KIFKTNICCFLVGIPRIIYGFRDNNFILSVEEFSTEVVLLKNNNPQLGNSCVDAIKWYGULTEWI UTRail 254 KMFKTNICCFLVGIPRIIYGFRDNNFILSVEEFSTEVVLLKNNNPQLGNSCVDAIKWYGULTEWI VGSACLEAIKWYGUFWYYGFRDNFILSSVEEFSTEVVLLKNNNPQLGNSCVDAIKWYGUFTEWI UTRail   255 KMFKTNICCFLVGIPRIYGFRDQVPKVYGFRDQVMLKTVEEFSTEEIPVILKNNNPTLGQSCLDAIKWYGLFTEWI MmDX0 273   273 KLLKWMAGSFLPGVPHVVAGFRNPEGFVCSLKTPPMMEMFENVNDREGWNPSVCMNFCAAFLSFI motif V motif VI	C.ELPRG NTTVDKK LQEIPRD KQTVKQH LKMIPRD LNQIPRD LNQIPRD LNSIPRE LKIIPRE
s.s. β15 β16 αΙ	
AgRail 326 PEDDFKLYRLSCSRGALHLRQLHDEDLANGDDITFGWFREWRRSISKSHGS   CaRail 343 DEIKSYRLKYDPVRKSFTLSETDSETNEKLRNGQ.LLTPEFTEWRQSLKK   SsRail 348 DTSKAYRVSFDPSTRTFTLRELMGNENSRLRNGG.LLTPEFTEWRQSLKK   SpRail 331 PPSTEFTLSYTGGSKLVLRQII   scrail 335 EDPHSQIRAFKLVFENNHLRLSEIEESDEYSGLIDGEHLISNGFKEWRRSLK   vpRail 326 DP.SIKKAYKLVFENNHLRLTEITEEGEFNELINGET   Ltrail 329 ES.AEHIRAYRLTFENNHLKVEIEKSDTEYDQLVNGEGLITSEFREWRKSLNKSL   cgrail 340 QSGNIRAYRLVYENNHLRLSEIEEQDGEYSRYVLEDGLITPEFKEWRESLRVPSEETPKAD	

**Supplementary Fig. 1.** Sequence alignment of fungal Rai1 homologs and mouse DXO (MmDXO). The secondary structure elements in the AgRai1 structure are shown (S. S.), and the six conserved sequence motifs are labeled. Strictly conserved residues among the aligned sequences are highlighted in red, mostly conserved residues in red. Residues that were selected for mutagenesis studies in AgRai1 and CaRai1 are highlighted in yellow.



**Supplementary Fig. 2.** Overlay of the structure of AgRai1 in a ternary complex with pU(S)6 and  $Mn^{2+}$  (in color) with that of free AgRai1 (gray).



**Supplementary Fig. 3.** Overlay of the structure of CaRai1 in a ternary complex with pU5 and  $Mn^{2+}$  (in color) with that of SsRai1 (gray).



**Supplementary Fig. 4.** Overlay of the active site region of the two molecules of CaRai1 in the crystallographic asymmetric unit of the ternary complex with pU5 and  $Mn^{2+}$ .