

Supplementary Table 1: Quantitation of Fig. 3A

Rai1 Exonuclease Activities

| | Time (min) | m⁷GpppRNA | % RNA remaining | GpppRNA | % RNA remaining | pppRNA | % RNA remaining | pRNA | % RNA 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

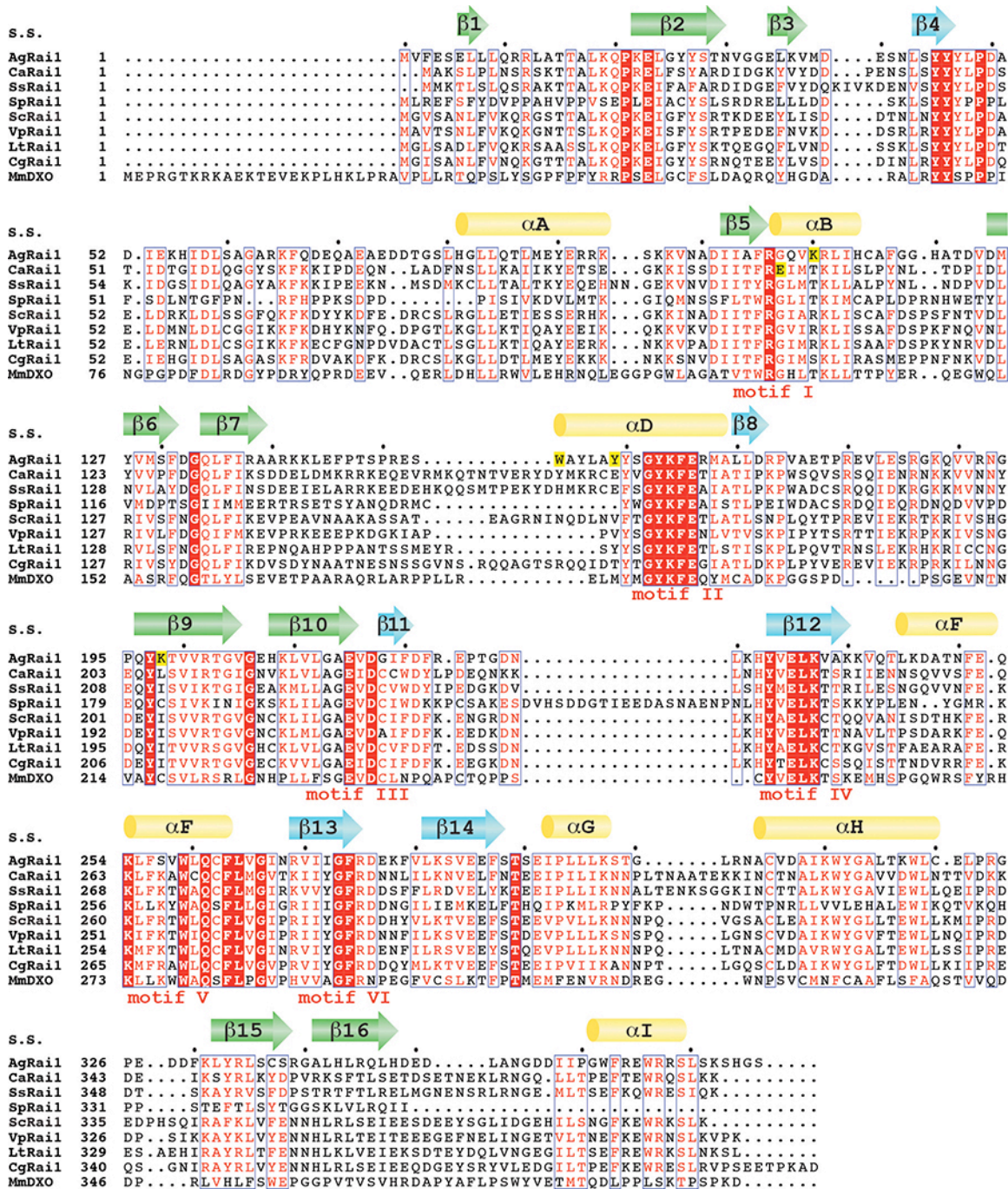
Rai1 Exonuclease Activities

| | Time (min) | m⁷GpppRNA | % RNA remaining | GpppRNA | % RNA remaining | pppRNA | % RNA remaining | pRNA | % RNA remaining |
|-------------------------------------|------------|-----------------------------|-----------------|----------------|-----------------|---------------|-----------------|-------------|-----------------|
| AgRai1 Wt | 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% |
| AgRai1 E215A/D217A | 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% |

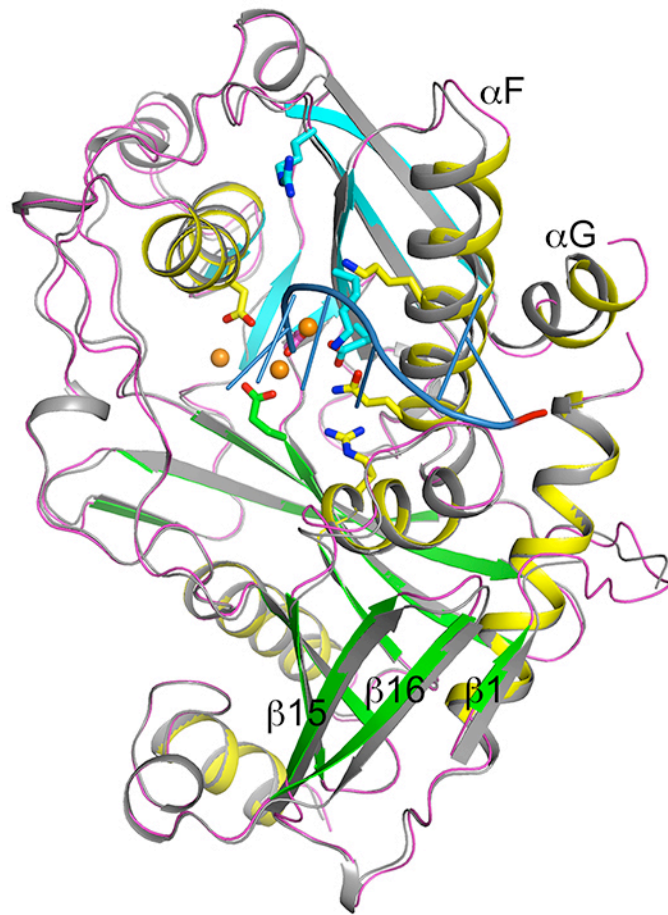
Supplementary Table 3: Quantitation of Fig. 6C

Rai1 and mutants Exonuclease Activities

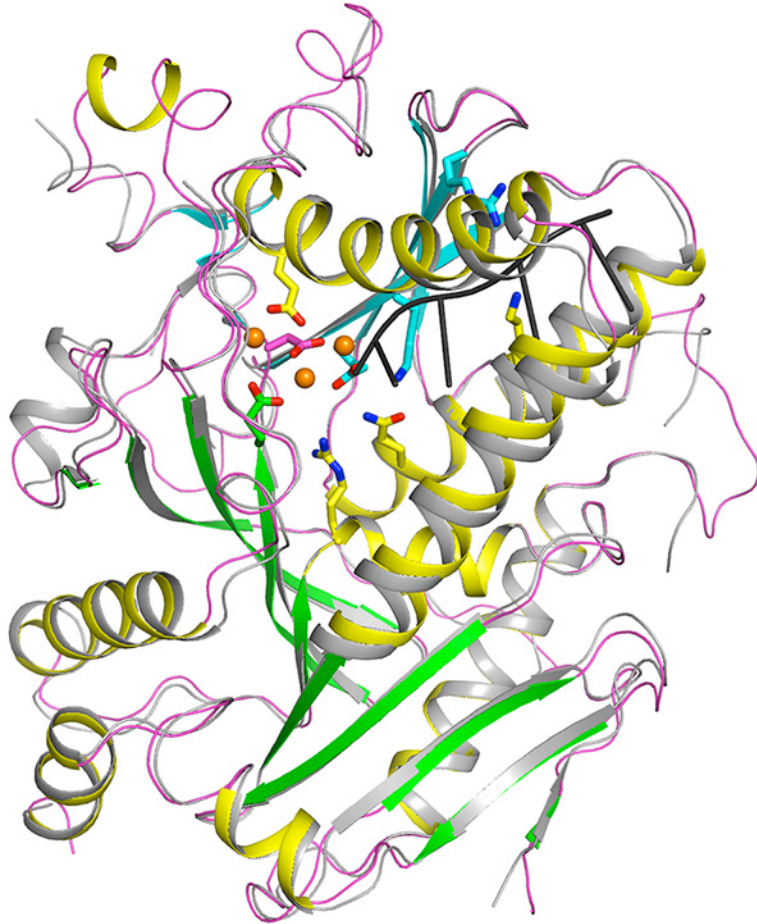
| | Time (min) | m ⁷ GpppRNA | % RNA remaining | pppRNA | % RNA remaining | pRNA | % RNA remaining | OH-RNA | % RNA remaining |
|---------------------------------|------------|------------------------|-----------------|--------|-----------------|-------|-----------------|--------|-----------------|
| 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 W154A/Y159E | 0 | 88925 | 100% | 76338 | 100% | 85540 | 100% | 76028 | 100% |
| | 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 E154A/Y159E K110T | 0 | 83542 | 100% | 76464 | 100% | 74862 | 100% | 76536 | 100% |
| | 20 | 85104 | 102% | 74044 | 97% | 80142 | 107% | 76864 | 100% |
| | 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% |



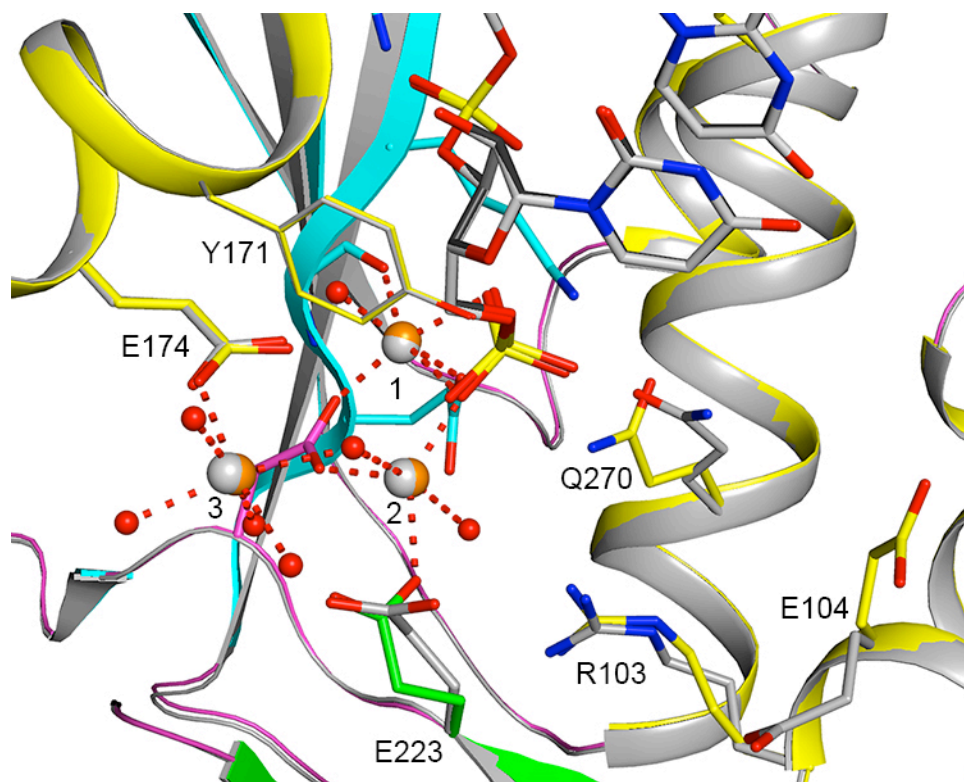
Supplementary Fig. 1. Sequence alignment of fungal Rai1 homologs and mouse DXO (MmDXO). The secondary structure elements in the AgRail structure are shown (S. S.), and the six conserved sequence motifs are labeled. Strictly conserved residues among the aligned sequences are highlighted in red, mostly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



Supplementary Fig. 2. Overlay of the structure of AgRai1 in a ternary complex with pU(S)6 and Mn²⁺ (in color) with that of free AgRai1 (gray).



Supplementary Fig. 3. Overlay of the structure of CaRai1 in a ternary complex with pU5 and Mn²⁺ (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 Mn²⁺.