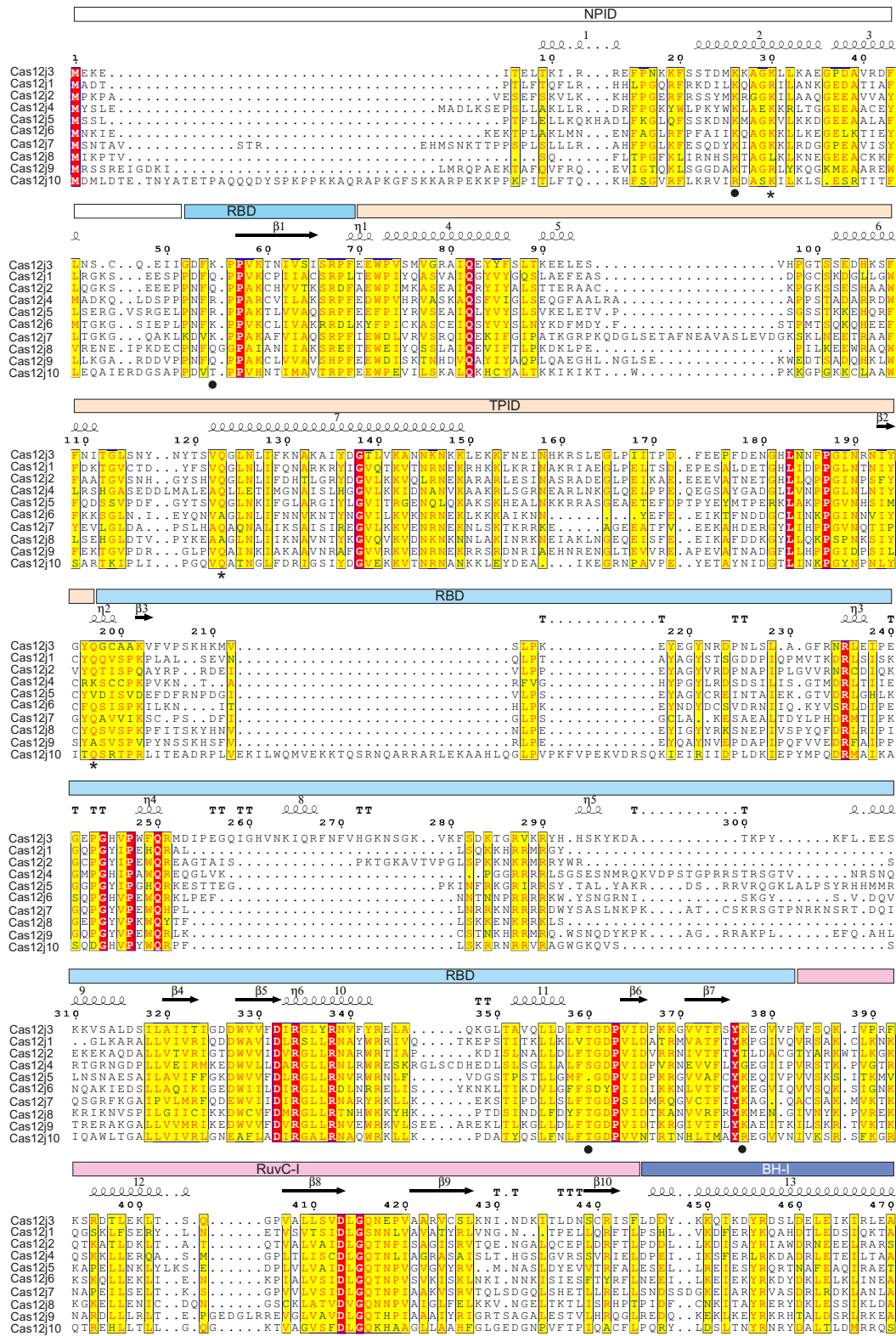


Supplementary. Fig. 1

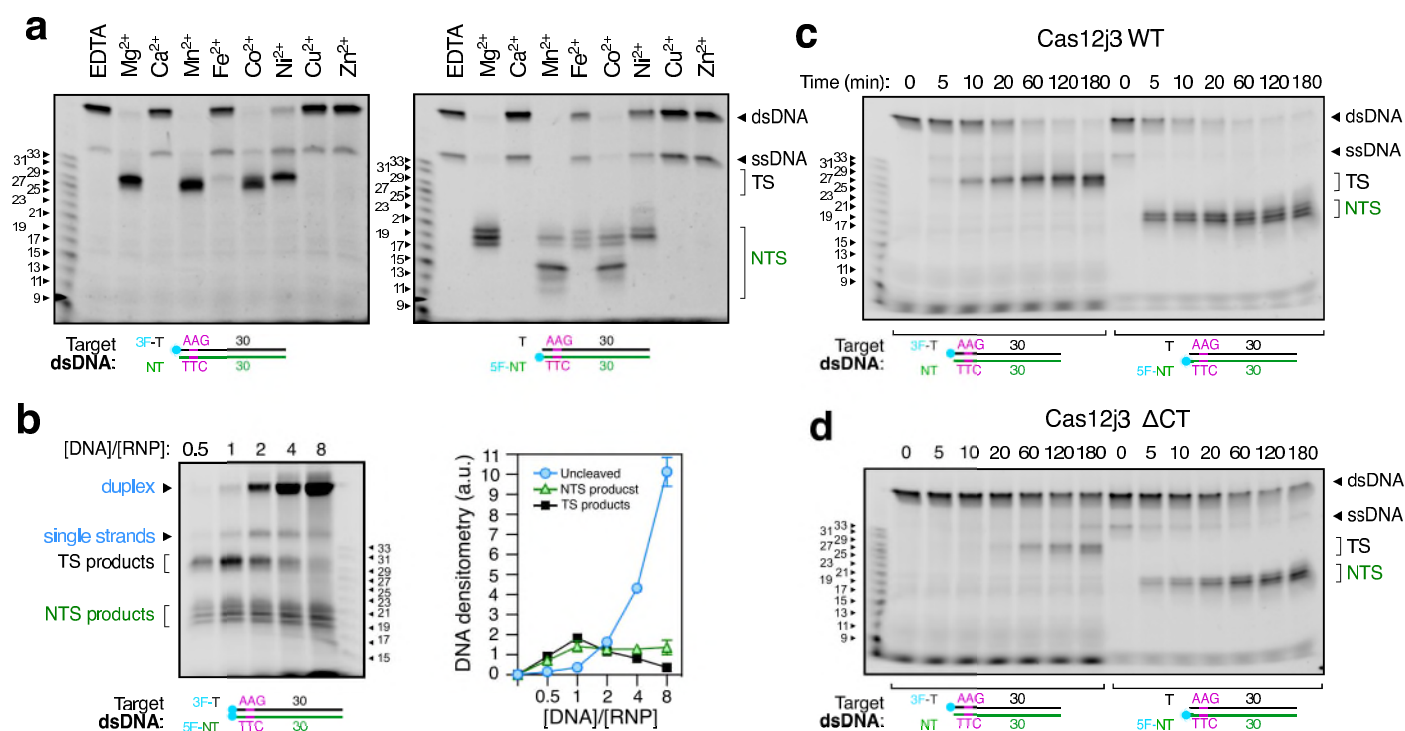




- \* PAM mutants
- Unwinding mutants
- ★ Catalytic mutants
- RuvC insertion mutants
- # STP and Plug mutants

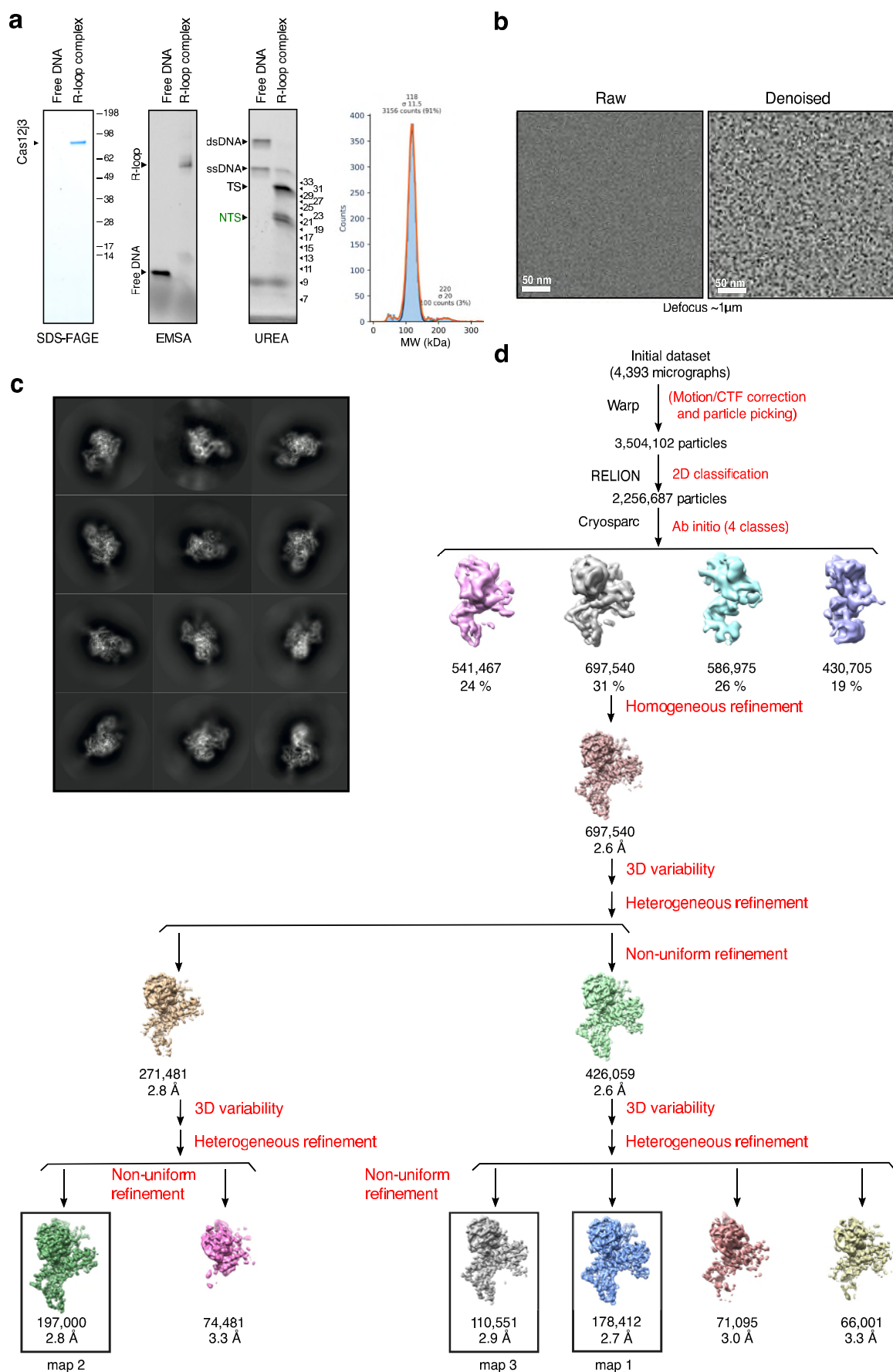
**Supplementary Figure 1.- Sequence alignment of known members of the Cas12j family.** The amino acid sequences of Cas12j 1 to 10 were aligned by Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo>). The figure was prepared with ESPrnt (<http://esprnt.ibcp.fr>). Residue numbers are labelled according to the Cas12j3 sequence. Similar residues are shown in red and identical residues in white over red background. The different structural domains of Cas12j3 and their amino acid composition are depicted as boxes above the sequences and labelled with the same names and colour code as in Fig. 2a. The mutations performed in key residues commented through the text are marked below.

Supplementary Fig. 2



**Supplementary Figure 2.- Cas12j3 endonuclease biochemical characterisation.** **a)** DNA cleavage dependency on divalent metal ions.  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Fe^{2+}$ ,  $Co^{2+}$  and  $Ni^{2+}$  metal ions support Cas12j3 catalytic activity, while  $Ca^{2+}$ ,  $Cu^{2+}$ ,  $Zn^{2+}$  do not. Depletion of the cation by EDTA abrogates phosphodiester hydrolysis. **b)** Cleavage assay using the target dsDNA shows the cleavage products of the different strands at different enzyme and substrates ratios. Quantification of the cleaved and non-cleaved dsDNA substrate is shown in the chart as mean  $\pm$  s.d. (see Methods). The curve shows an increase of the non-cleaved substrate when a 1:1 ratio is reached. An asymptotic behaviour is observed for the NT-strand products. **c)** Time course of the cleavage reaction by Cas12j3. Cas12j3 endonuclease completes the reaction in approximately 120 min for the T-strand while the NT-strand cleavage is completed in 20 min. **d)** Time course of the cleavage reaction by Cas12j3- $\Delta$ CT mutant lacking the C-terminal 39 residues. Oligonucleotides 3F-T-AAG-30/NT-TTC-30, T-AAG-30/5F-NT-TTC-30 and 3F-T-AAG-30/5F-NT-TTC-30 (Supplementary Table 2) were used as substrate to visualize the cleavage of the target, non-target and both DNA strands in a-d). Experiments displayed are representative of three replicates. DNA markers are shown in nucleotides.

## Supplementary. Fig. 3

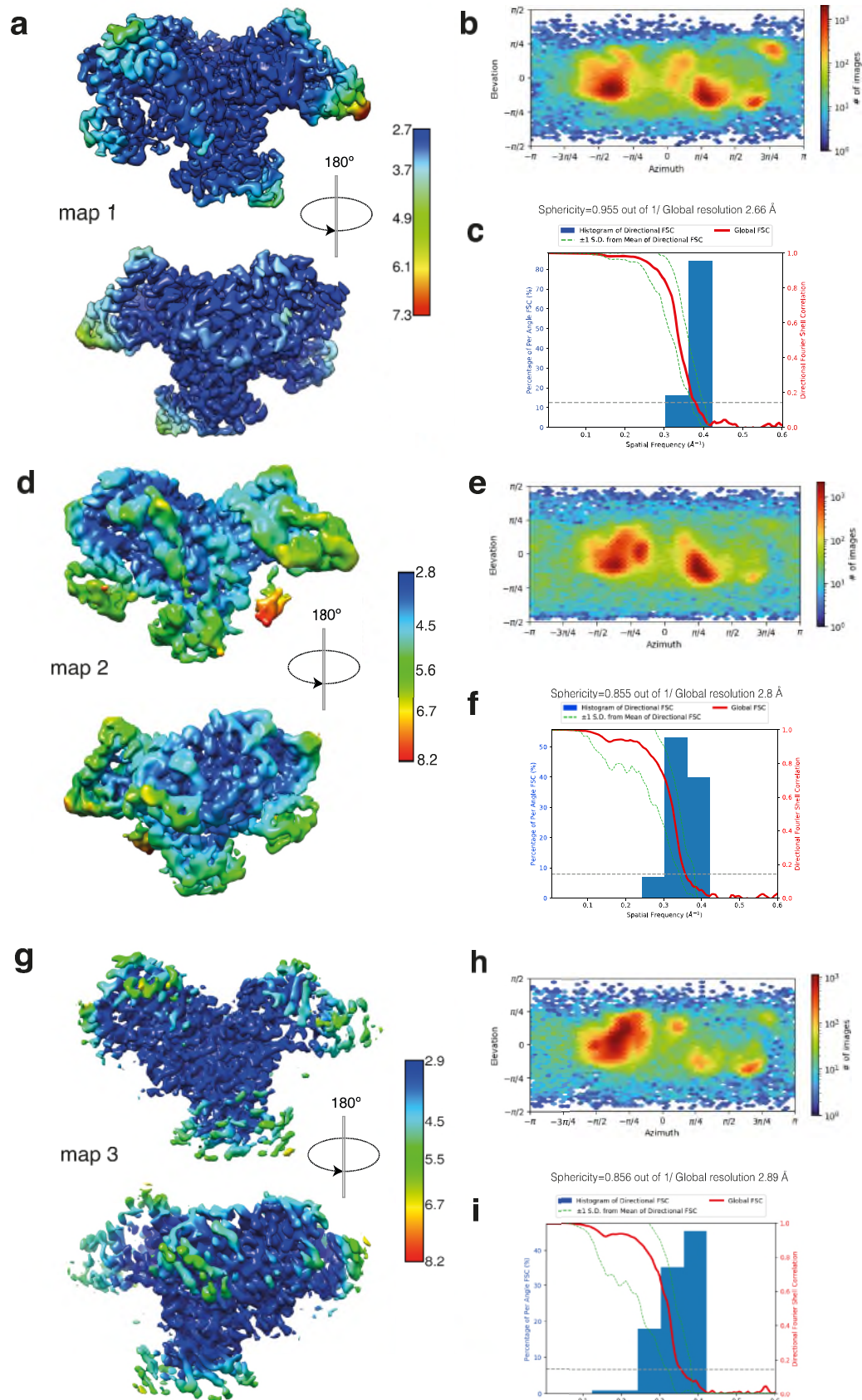


**Supplementary Figure 3.- Single particle cryo-EM analysis of the Cas12j3 endonuclease R-loop complex. a)** SDS-PAGE, electrophoretic mobility shift assay (EMSA), and denaturing polyacrylamide gels showing the reconstitution of Cas12j3/R-loop complex. The complex shown in a) was reconstituted with the labeled oligonucleotides 3F-T-AAG-30/5F-NT-TTC-30 under similar conditions as the complex used for

cryo-EM. The right-side panel shows the experimental mass and homogeneity of the complex used for cryo-EM (i.e., using the unlabeled nucleotides T-AAG-30/NT-TTC-30), estimated by mass photometry. The position of the protein markers is shown in the SDS-PAGE gel in kDa. The position of the DNA markers is shown in the UREA gel in nucleotides. **b)** Representative cryo-EM micrograph of the Cas12j3/R-loop complex in vitreous ice on gold grids before and after denoising with WARP<sup>1</sup>. Similar particle distribution and ice quality was observed along the 4,393 micrographs used during the processing, and in similar datasets that were not used in the processing. **c)** Representative reference-free 2D class averages sorted by class distribution (RELION<sup>2</sup>). **d)** Overview of the cryo-EM data processing workflow for the Cas12j3/R-loop complex. Similar results of the data processing were obtained with another dataset; however, the ice quality was worse, and it yielded a lower resolution map.



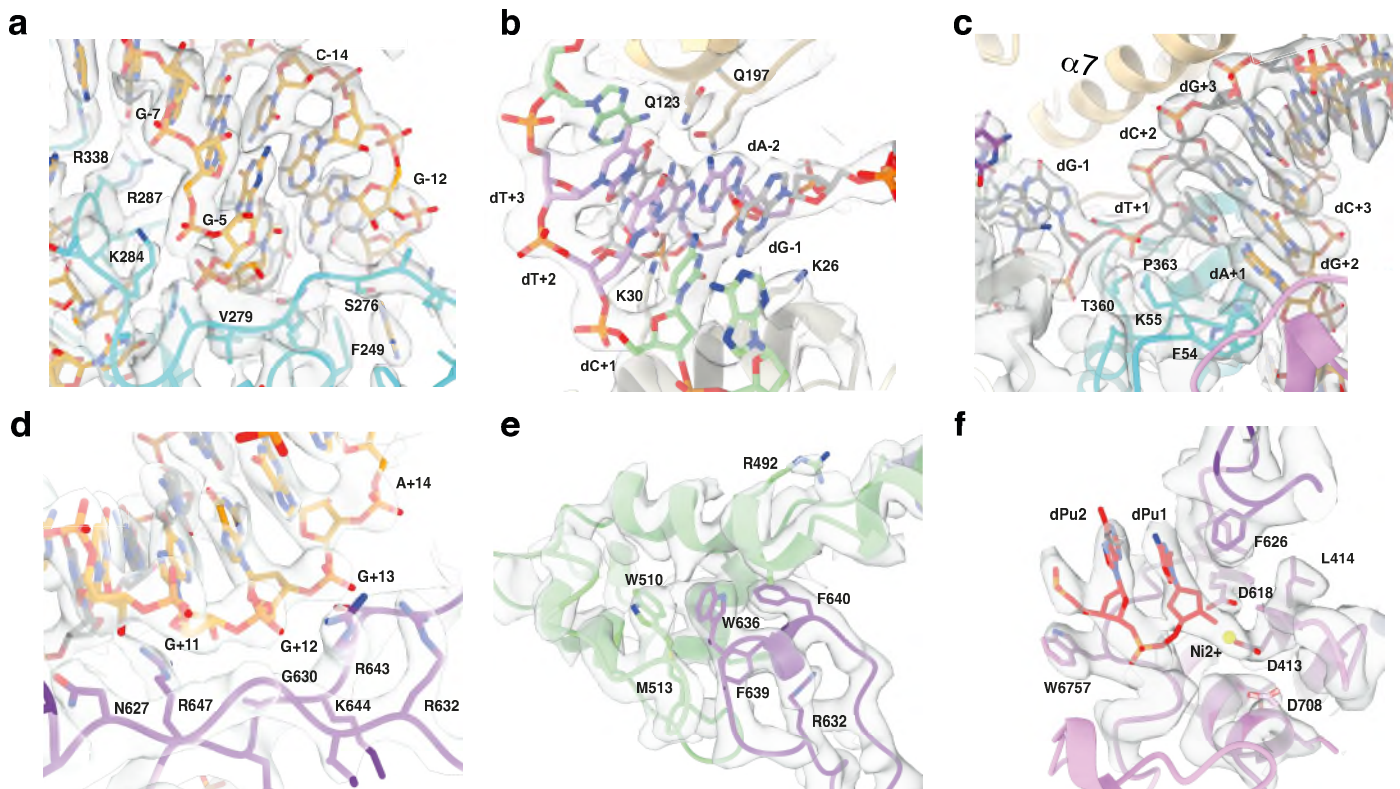
Supplementary. Fig. 4



**Supplementary Figure 4.- Resolution assessment, validation of cryoEM density maps. a, d, g) Local resolution cryo-EM maps of the Cas12j3-R-loop complex. b, e, h) Angular distribution plot showing the**

relative orientation of particles in the final 3D reconstruction. **c, f, i**) Fourier shell correlation (FSC) curves and sphericity of the final cryo-EM density maps (3D-FSC<sup>3</sup>, Supplementary Table 1).

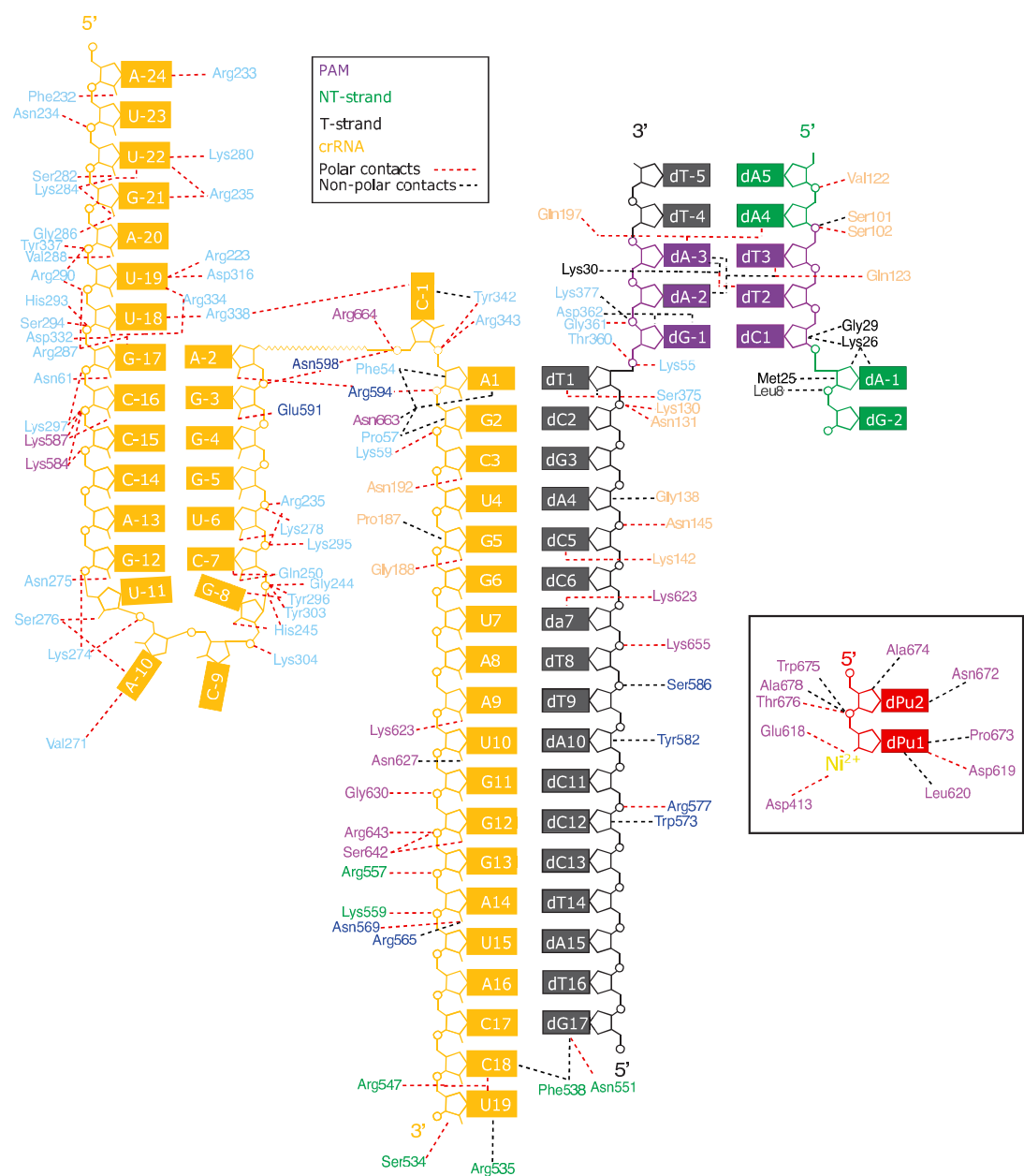
Supplementary. Fig. 5



**Supplementary Figure 5.- Fit of the model to map 1 in specific regions.** **a)** View of the high-resolution cryo-EM map in the RBD domain (contour level 7.5), **b)** PAM binding region (contour level 7.0), **c)** unzipping cavity (contour level 7.5), **d)** RuvC insertion (contour level 7.8), **e)** hydrophobic plug (contour level 6) and **f)** the RuvC catalytic site (contour level 5.7).

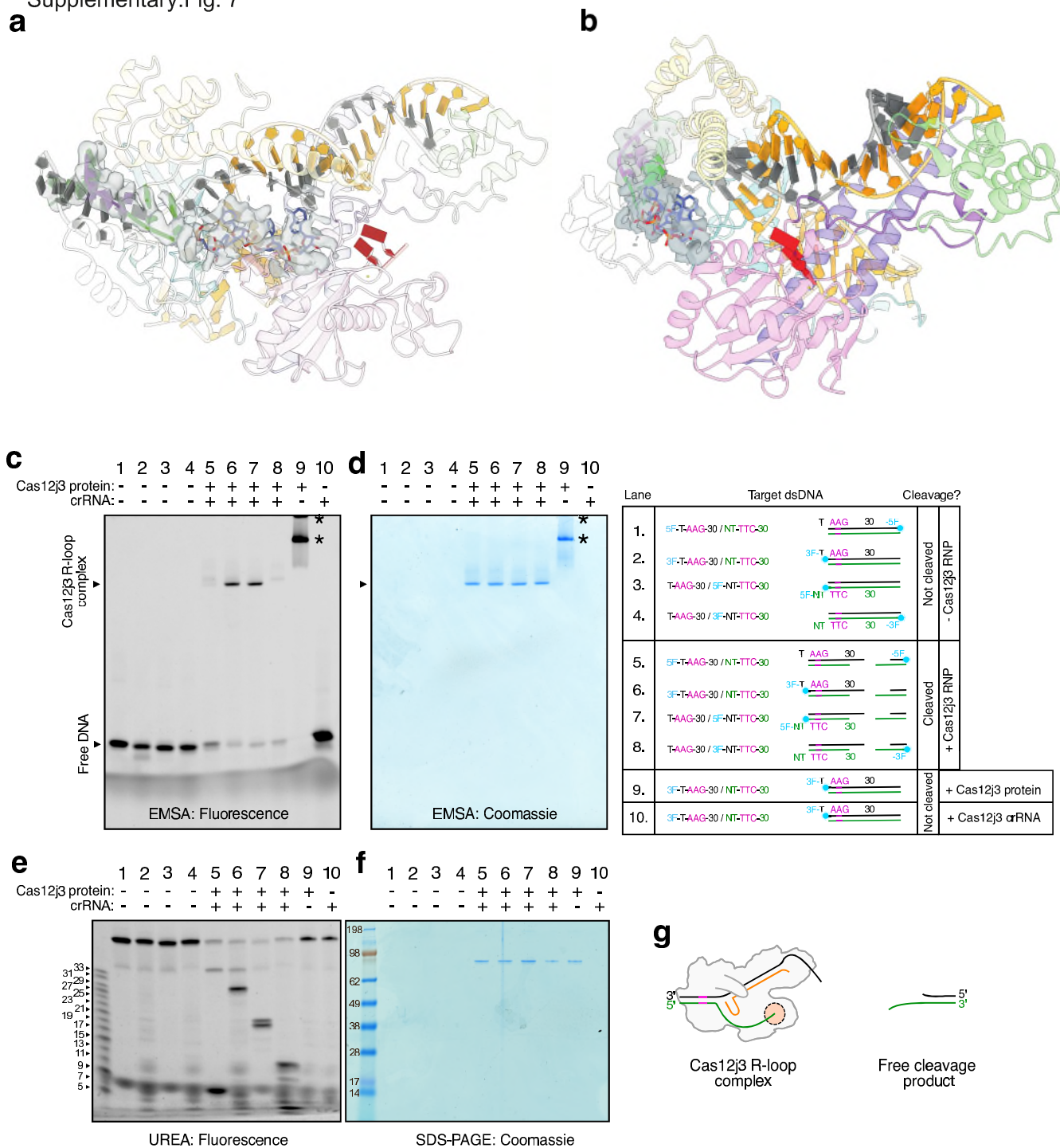


Supplementary. Fig. 6



**Supplementary Figure 6.- Protein-nucleic acids interactions in the Cas12j3-R-loop structure.** Polar and non-polar contacts of the nucleic acids with the protein side and main chain are indicated (see key in the figure).

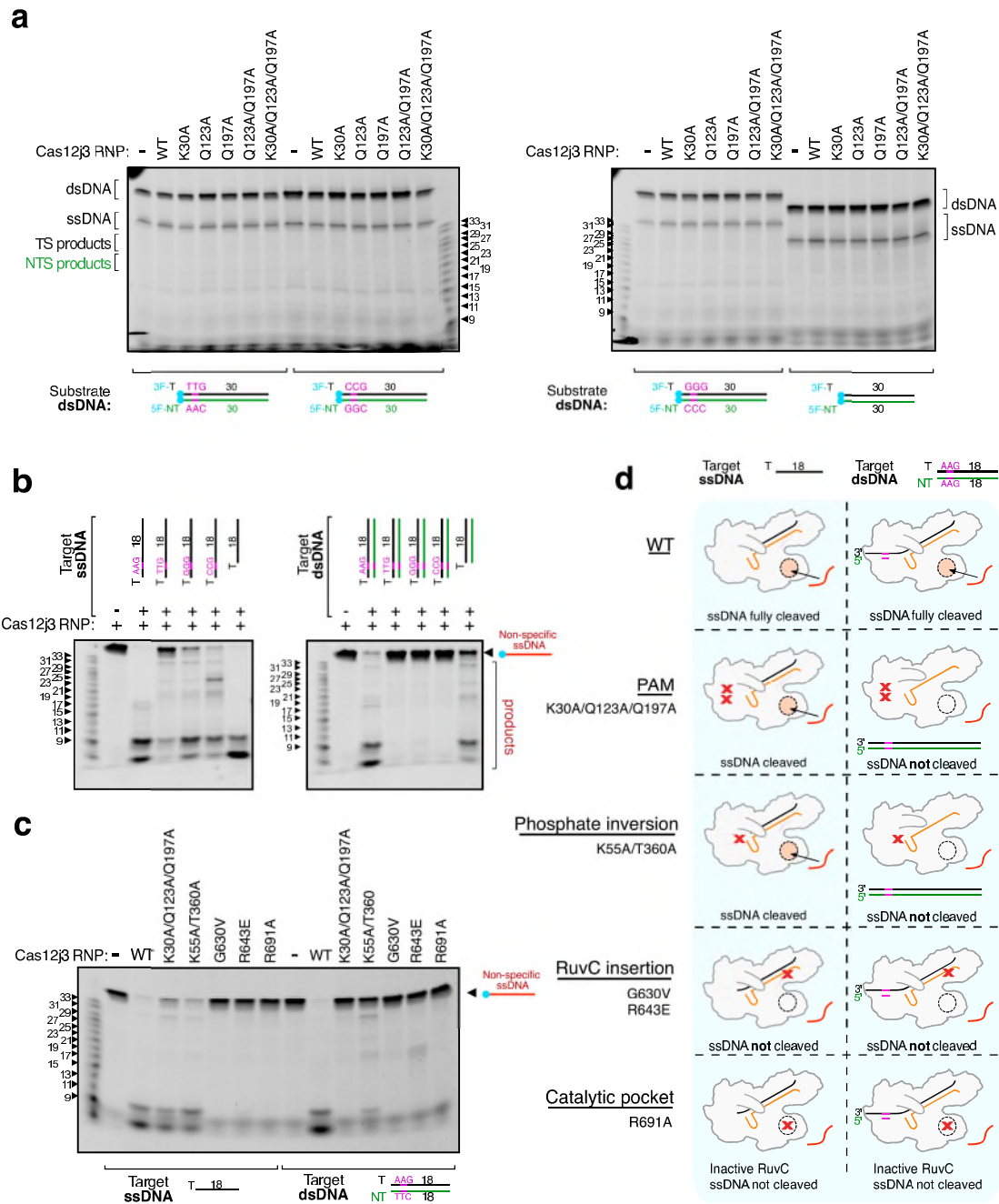
Supplementary.Fig. 7



**Supplementary Figure 7.- The cleaved NT-strand of the Cas12j3-R-loop complex. a-b)** Two different views of the cryo-EM map density observed at low contour suggesting that the dinucleotide in the catalytic

site belongs to the NT-strand. The nucleotides in blue are shown in a) for illustrative purposes only. **c-f)** Binding assay using the NT- and T-strands, FAM labelled in the 5' and 3' positions (for clarity, see table on the right). Lanes 1-4 represent the four free DNAs. Lanes 5-8 show correspond to the R-loop complexes generated after cleavage. Lane 9 corresponds to unspecific complexes (\*) formed by the protein and the DNA (no crRNA). Lane 10 is a control experiment including the target dsDNA and the crRNA (no Cas12j3 protein). c) EMSA (electrophoretic mobility shift assay) gel scanned for the FAM fluorescent signal. d) Same gel as shown in c) stained with Coomassie. e) denaturing polyacrylamide gel of the same samples as shown in a) scanned for the FAM fluorescent signal. In e) DNA markers are shown in nucleotides. f) SDS-PAGE of the samples as shown in a) stained with Coomassie. In f) Protein markers are shown in kDa **g)** Cartoon representation of the interpretation of the experiments shown in c-f). After cleavage of the target dsDNA, the PAM-distal fragments are not bound to the complex (lanes 5 and 8), while the PAM-proximal strands remain bound in the R-loop structure. The results shown in c-f) are representative of 3 independent experiments with similar results.

## Supplementary Fig. 8.



**Supplementary Figure 8.- PAM specificity and crRNA/DNA hybrid assembly. a)** cleavage assay with Cas12j3 WT and PAM interacting mutants, using target dsDNA as substrate containing different PAM or no PAM sequence. Oligonucleotides 3F-T-AAG-30/5F-NT-TTC-30, 3F-T-TTG-30/5F-NT-AAC-30, 3F-T-CCG-30/5F-NT-GGC-30, 3F-T-GGG-30/5F-NT-CCC-30 and 3F-T-30/5F-NT-30 were used as activators (Supplementary Table II) **b)** Cas12j3 activation of unspecific ssDNA degradation assay using an 18-nt dsDNA

containing different PAM or no PAM sequence as activator. **c)** Unspecific ssDNA degradation by Cas12j3 WT and representative mutants involved in the PAM recognition (K30A/Q123A/Q197A), unwinding (K55A), and RuvC insertion (R643A) after activation with a 18-nt ssDNA without the PAM or a 18-nt dsDNA with the PAM. **d)** schematic representation explaining the results of the experiments shown in c). Gels shown are representative of three independent experiments with similar results. The DNA markers are shown in nucleotides.



## Supplementary Tables

**Supplementary Table 1. Cryo-EM data collection, refinement and validation statistics**

|                                                  | EMDB-12827<br>map1<br>PDB 7ODF | EMDB-12827<br>map2 | EMDB-12827<br>map3 |
|--------------------------------------------------|--------------------------------|--------------------|--------------------|
| <b>Data collection and processing</b>            |                                |                    |                    |
| Voltage (kV)                                     | 300                            | 300                | 300                |
| Total electron exposure (e-/Å <sup>2</sup> )     | 42 (1.05/frame)                | 42 (1.05/frame)    | 42 (1.05/frame)    |
| Defocus range (μm)                               | 0.8/2.6                        | 0.8/2.6            | 0.8/2.6            |
| Pixel size (Å)                                   | 0.832                          | 0.832              | 0.832              |
| Symmetry imposed                                 | n/a                            | n/a                | n/a                |
| Initial particle images (no.)                    | 3,504,102                      | 3,504,102          | 3,504,102          |
| Final particle images (no.)                      | 178,412                        | 197,000            | 110,551            |
| Sphericity                                       | 0.955                          | 0.855              | 0.856              |
| Map resolution (Å)                               | 2.66                           | 2.80               | 2.84               |
| FSC threshold                                    | 0.143                          | 0.143              | 0.143              |
| Map resolution range (Å)                         | 2.66-10.19                     | 2.8-11.53          | 2.87-11.13         |
| <b>Refinement</b>                                |                                |                    |                    |
| Initial model used (PDB code)                    | none                           |                    |                    |
| Model resolution (Å)                             | 2.92                           |                    |                    |
| FSC threshold                                    | 0.143                          |                    |                    |
| Model resolution range (Å)                       | 2.7-7.3                        |                    |                    |
| Map sharpening <i>B</i> factor (Å <sup>2</sup> ) | -67.26                         |                    |                    |
| Model composition                                |                                |                    |                    |
| Non-hydrogen atoms                               | 6847                           |                    |                    |
| Protein residues                                 | 703                            |                    |                    |
| Nucleotides                                      | 78                             |                    |                    |
| Ligands                                          | Ni:1 Zn:1                      |                    |                    |
| Water                                            | 5                              |                    |                    |
| <i>B</i> factors (Å <sup>2</sup> )               | Min/max/mean                   |                    |                    |
| Protein                                          | 50.00/144.89/80.86             |                    |                    |
| Nucleotide                                       | 20.00/142.61/79.35             |                    |                    |
| Ligand                                           | 99.25/127.66/113.45            |                    |                    |
| Water                                            | 80.64/80.64/80.64              |                    |                    |
| R.m.s. deviations                                |                                |                    |                    |
| Bond lengths (Å)                                 | 0.004 (0)                      |                    |                    |
| Bond angles (°)                                  | 0.827 (0)                      |                    |                    |
| Validation                                       |                                |                    |                    |
| MolProbity score                                 | 1.35                           |                    |                    |
| Clashscore                                       | 6.44                           |                    |                    |
| Rotamer outliers (%)                             | 0.78                           |                    |                    |
| CaBLAM outliers (%)                              | 0.72                           |                    |                    |
| Cβ outliers (%)                                  | 0.0                            |                    |                    |
| Peptide plane (%)                                |                                |                    |                    |
| Cis proline/general                              | 0.0/0.0                        |                    |                    |
| Twisted proline/general                          | 0.0/0.0                        |                    |                    |
| Ramachandran plot                                |                                |                    |                    |
| Favored (%)                                      | 98.57                          |                    |                    |
| Allowed (%)                                      | 1.43                           |                    |                    |
| Disallowed (%)                                   | 0.0                            |                    |                    |

**Supplementary Table 2.- Oligonucleotides and primer sequences used in this study**

| Oligonucleotides used for Cleavage assays |                                                 |
|-------------------------------------------|-------------------------------------------------|
| Name                                      | Sequence (5' to 3')                             |
| 3F-T-AAG-30                               | GCTGTAGCACAAAGGTATCCCATTACCAGCTGAATTAC/36-FAM/  |
| 5F-NT-TTC-30                              | /56-FAM/GTAATTTCAGCTGGTAATGGGATACCTTGTGCTACAGC  |
| T-AAG-30-5F                               | /56-FAM/GCTGTAGCACAAAGGTATCCCATTACCAGCTGAATTAC  |
| NT-TTC-30-3F                              | GTAATTTCAGCTGGTAATGGGATACCTTGTGCTACAGC/36-FAM/  |
| T-AAG-30                                  | GCTGTAGCACAAAGGTATCCCATTACCAGCTGAATTAC          |
| T-AAG-27                                  | GTAGCACAAAGGTATCCCATTACCAGCTGAATTAC             |
| T-AAG-24                                  | GCACAAGGTATCCCATTACCAGCTGAATTAC                 |
| T-AAG-21                                  | CAAGGTATCCCATTACCAGCTGAATTAC                    |
| T-AAG-18                                  | GGTATCCCATTACCAGCTGAATTAC                       |
| T-AAG-15                                  | ATCCCATTACCAGCTGAATTAC                          |
| T-AAG-12                                  | CCATTACCAGCTGAATTAC                             |
| T-AAG-9                                   | TTACCAGCTGAATTAC                                |
| T-AAG-6                                   | CCAGCTGAATTAC                                   |
| T-AAG-3                                   | GCTGAATTAC                                      |
| NT-TTC-30                                 | GTAATTTCAGCTGGTAATGGGATACCTTGTGCTACAGC          |
| NT-TTC-27                                 | GTAATTTCAGCTGGTAATGGGATACCTTGTGCTAC             |
| NT-TTC-24                                 | GTAATTTCAGCTGGTAATGGGATACCTTGTGC                |
| NT-TTC-21                                 | GTAATTTCAGCTGGTAATGGGATACCTTG                   |
| NT-TTC-18                                 | GTAATTTCAGCTGGTAATGGGATACC                      |
| NT-TTC-15                                 | GTAATTTCAGCTGGTAATGGGAT                         |
| NT-TTC-12                                 | GTAATTTCAGCTGGTAATGG                            |
| NT-TTC-9                                  | GTAATTTCAGCTGGTAA                               |
| NT-TTC-6                                  | GTAATTTCAGCTGG                                  |
| NT-TTC-3                                  | GTAATTTCAGC                                     |
| 3F-T-TTG-30                               | GCTGTAGCACAAAGGTATCCCATTACCAGCTGTTTAC/36-FAM/   |
| 5F-NT-AAC-30                              | /56-FAM/GTAAAACAGCTGGTAATGGGATACCTTGTGCTACAGC   |
| 3F-T-CCG-30                               | GCTGTAGCACAAAGGTATCCCATTACCAGCTGCCATTAC/36-FAM/ |
| 5F-NT-GGC-30                              | /56-FAM/GTAAAGCAGCTGGTAATGGGATACCTTGTGCTACAGC   |
| 3F-T-GGG-30                               | GCTGTAGCACAAAGGTATCCCATTACCAGCTGGGTTAC/36-FAM/  |
| 5F-NT-CCC-30                              | /56-FAM/GTAAACCAGCTGGTAATGGGATACCTTGTGCTACAGC   |
| 3F-T-30                                   | GCTGTAGCACAAAGGTATCCCATTACCAGCT/36-FAM/         |
| 5F-NT-30                                  | /56-FAM/AGCTGGTAATGGGATACCTTGTGCTACAGC          |
| T-TTG-18                                  | GGTATCCCATTACCAGCTGTTTAC                        |
| NT-AAC-18                                 | GTAAAACAGCTGGTAATGGGATACC                       |
| T-GGG-18                                  | GGTATCCCATTACCAGCTGGGTTAC                       |
| NT-CCC-18                                 | GTAACCAGCTGGTAATGGGATACC                        |
| T-CCG-18                                  | GGTATCCCATTACCAGCTGCCATTAC                      |
| NT-GGC-18                                 | GTAAGGCAGCTGGTAATGGGATACC                       |
| T-18                                      | GGTATCCCATTACCAGCT                              |
| NT-18                                     | AGCTGGTAATGGGATACC                              |

|                                                                        |                                                   |
|------------------------------------------------------------------------|---------------------------------------------------|
| Non-Specific ssDNA                                                     | /56-FAM/ATTTAAATAGGGAAGATAAGCAAAGGGTTGACGAAAGCC   |
| crRNA                                                                  | UAUUGAUUGCCCAGUACGCGUGGGACAGCUGGUAAUGGGAUACCUU    |
| <b>Oligonucleotides used for cloning and site-directed mutagenesis</b> |                                                   |
| Name                                                                   | Sequence (5' to 3')                               |
| K26A-For                                                               | CGATATGGCAAAAGCGGGCAAACCTGCTGAAAGCGGAAG           |
| K26A-Rev                                                               | CCCCTTTTGGCCATATCGGTGCTGCTAAATTTTGTTCGGAAATTCGC   |
| K30A-For                                                               | ATGAAAAAAGCGGGCGCACTGCTGAAAGCGGAAGGCCCCG          |
| K30A-Rev                                                               | TCCGCTTTCAGCAGTGCGCCCGCTTTTTTCATATCGGTG           |
| K55A-For                                                               | TTGGCGATTTTGCACCGCCGGTGAAAACCAACATTGTGAGC         |
| K55A-Rev                                                               | ccggcgggtgcaaaatcgccaaTAATTTCTTGGCAG              |
| Q123A-For                                                              | AGCGTGGCGGGCCTGAACCTGATTTTTTAAAAACGCG             |
| Q123A-Rev                                                              | AGGCCCCGCCACGCTGGTATAGTTATAGTTGCTCAGGC            |
| Q197A-For                                                              | GGCTATGCGGGCTGCGCGGCGAAAG                         |
| Q197A-Rev                                                              | GCAGCCCGCATAGCCATAAATGTTGCGGTTAATGCCCG            |
| T360A-For                                                              | TGTTTGCCGCGGATCCGGTGATTGATCCGAAAAAAG              |
| T360A-Rev                                                              | GCCGGCAAACAGATCCAGCAGCTGCACC                      |
| K377A-For                                                              | AGCTATGCAGAAGGCGTGGTGCCGGTGT'TTAGCCAG             |
| K377A-Rev                                                              | CCTTCTGCATAGCTAAAGGTCACCACGCCTTTTTTCGGATCAATCACC  |
| D413A-For                                                              | AGCGTGGCCCTGGGCCAGAACGAACCGGTG                    |
| D413A-Rev                                                              | GCCCAGGGCCACGCTCAGCAGCGCCACC                      |
| E618A-For                                                              | TTATTCTGGCAGATCTGGATGTGAAAAAAAATTTAACGGCCGCG      |
| E618A-Rev                                                              | CAGATCTGCCAGAATAATCACAATATCGTTAATGCCGCTC          |
| G630A-For                                                              | GCCGCGGATTCGCGATATTGGCTGGGATAACTTTTTTAGCAG        |
| G630A-Rev                                                              | GCGAATCGCGCGGCCGTAAATTTTTTTTTTCACATCCAGATCTTCC    |
| R643E-For                                                              | GCAGCGAGAAAGAAAACCGCTGGTTTATTCGGCGGTTTC           |
| R643E-Rev                                                              | GTTTTCTTTCTCGCTGCTAAAAAAGTTATCCAGCCAATATCGCGAATGC |
| R691A-For                                                              | AAAACGCCGATGGCATTAACTTTACCTGCCGCAAATGC            |
| R691A-Rev                                                              | AATGCCATCGGCGTTTTCTTTGCTGCAAAAGCCGCAATCCG         |
| D708A-For                                                              | CTATCATGCGGCTATTGATGTGGCGACCCGTAACATTGCG          |
| D708A-Rev                                                              | CATCAATAGCCGCATGATAGCTCAGCCCGC                    |
| Cas12j3-ΔCT-For                                                        | AATCTGTACTTTCAAGGCAGCGGCCCGGCGG                   |
| Cas12j3-ΔCT-Rev                                                        | TTGAAAGTACAGATTTTCCATCGGTTTGCCAGCACCG             |

## cDNA sequence of Cas12j3

```

atggaaaaaagaattaccgaactgaccaaatttcgcgcggaattccgaacaaaaatttagcagcaccgatatgaaaaagcgggcaaaactgctgaaagcgggaagg
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**Supplementary Table 3.-** DALI search displaying the homology of Cas12j3 with other RuvC family domains.

| PDB code | rmsd (Å) | lali | nres | id % | Protein                                    |
|----------|----------|------|------|------|--------------------------------------------|
| 5u33-A   | 4.5      | 386  | 1085 | 11   | Cas12b                                     |
| 6xmf-A   | 5.3      | 384  | 627  | 13   | Cas12g                                     |
| 7c7l-A   | 4.3      | 325  | 503  | 17   | Cas12f                                     |
| 6w64-A   | 4.8      | 376  | 1037 | 12   | Cas12i                                     |
| 5xuu-A   | 5.2      | 347  | 1213 | 13   | Cas12a                                     |
| 56fa-A   | 2.6      | 113  | 130  | 16   | CNVP61<br>Holiday<br>Junction<br>resolvase |

## Supplementary References

- 1 Tegunov, D. & Cramer, P. Real-time cryo-electron microscopy data preprocessing with Warp. *Nature Methods* 16, 1146-1152, doi:10.1038/s41592-019-0580-y (2019).
- 2 Zivanov, J. et al. New tools for automated high-resolution cryo-EM structure determination in RELION-3. *Elife* 7, doi:10.7554/eLife.42166 (2018).
- 3 Tan, Y. Z. et al. Addressing preferred specimen orientation in single-particle cryo-EM through tilting. *Nat Methods* 14, 793-796, doi:10.1038/nmeth.4347 (2017).