A

Oligonucleotide Substrates	Sequence
ssU24	6-FAM 5’-TGTTGTGAGGAATGAAGTTGATTUAAATGTGATGAGGTGAGGGAG
ssU11	6-FAM 5’-GAAGTTGATTUAAATGTGATG
G-complement	5’-CTCCCTCACCTCATCACATTTGAATCAACTTCATTCCTCACAACA
A-complement	5’-CTCCCTCACCTCATCACATTTAAATCAACTTCATTCCTCACAACA

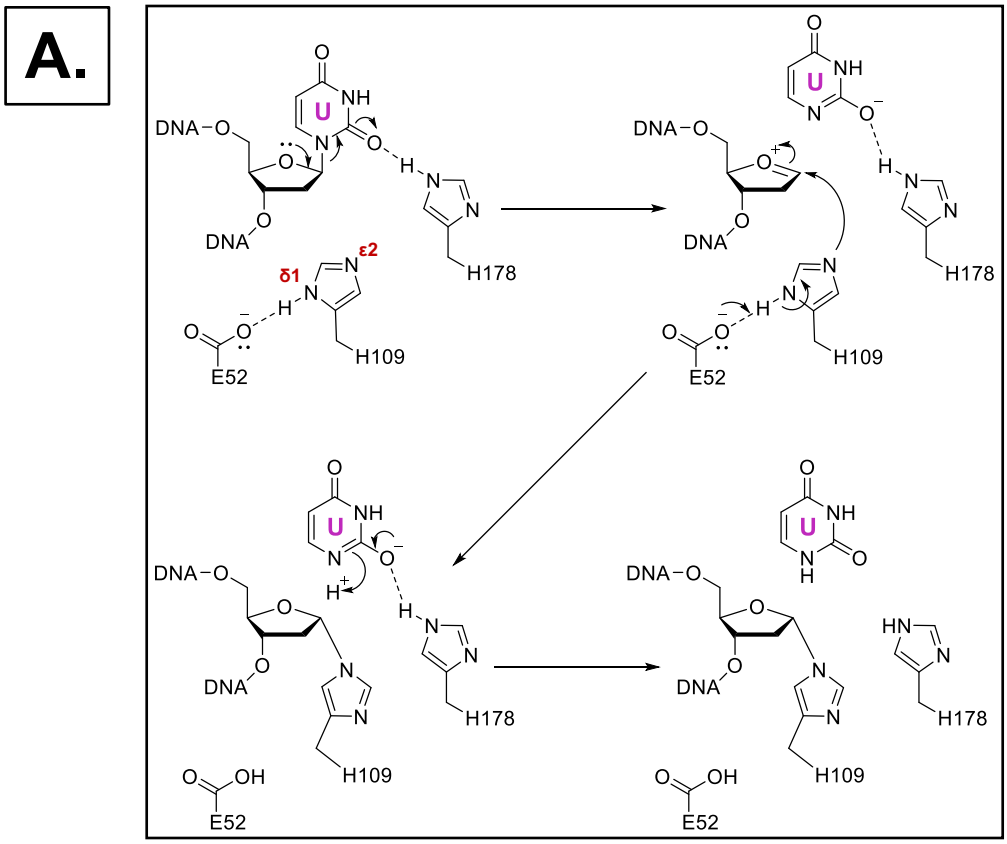
B

Primers	Sequence
SDS RBS Forward	5’-GGGAGACCCAAGCTGAGGAGCCACCATGGACTACAAAGAC
SDS RBS Reverse	5’-GTCTTTGTAGTCCATGGTGGCTCCTCAGCTTGGGTCTCCC
pHis-UdgX Forward	5'-GCCGCGGCCGCGGAGCCATATGGCGGGTGCGCAA
pHis-UdgX Reverse	5’-GCGCGCGGATCCTCATGGCCTGACATCTGCTGCGAC
SDM H109S Forward	5'-GGCAAACGACGCATCAGCAAGACCCCCAGTCG
SDM H109S Reverse	5'-CGACTGGGGGTCTTGCTGATGCGTCGTTTGCC

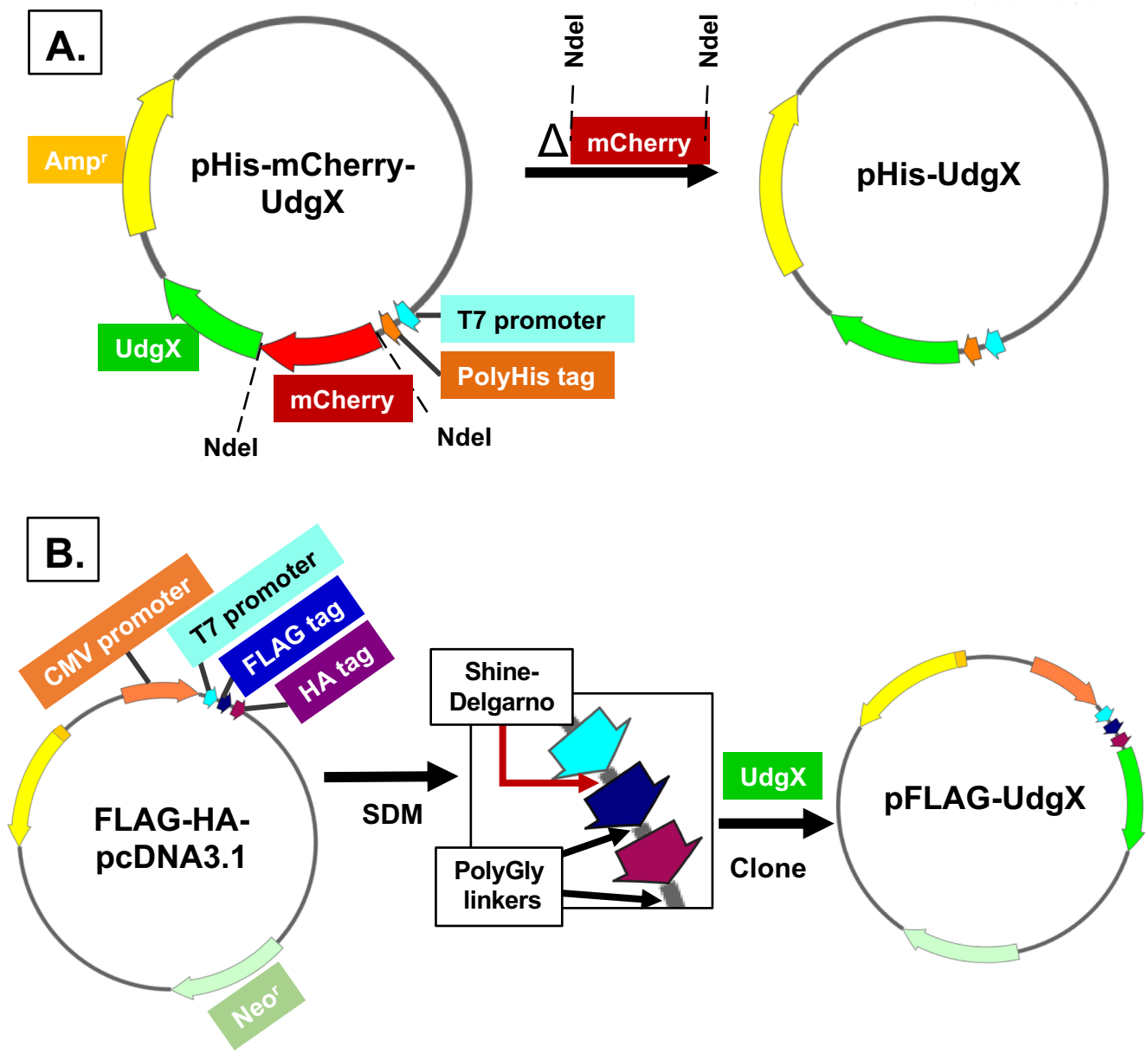
C

Oligonucleotide Substrates	Sequence
30-mer	6-FAM 5’-GCAAAAAGGGCAAGCTGTTCAAAAAAATGA

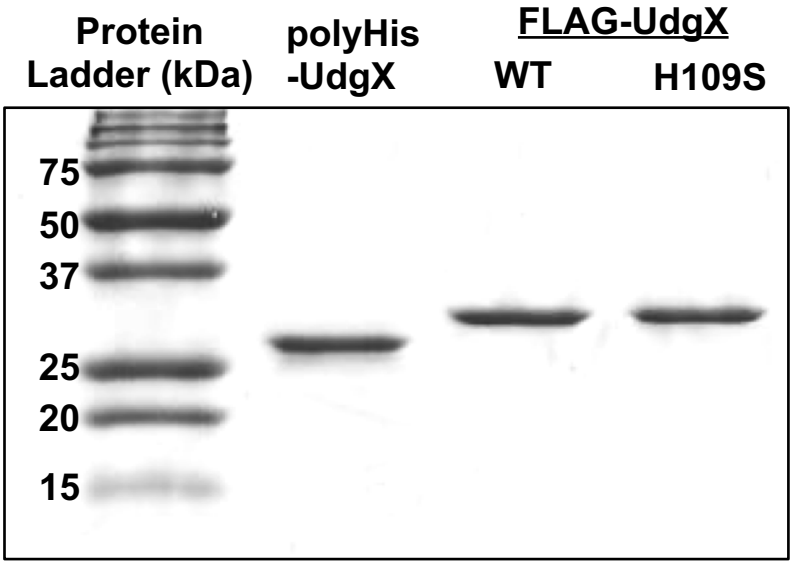
Supplementary Table 1. Sequences of synthetic substrates and primers. A. The uracil (red)-containing oligomers are modified at the 5’ end with a 6-Carboxyfluorescein (6-FAM) fluorophore. The complement strands used to generate dsDNA substrates have the guanine (blue) and adenine (green) indicated. B. Sequences of primers used for cloning and site-directed mutagenesis (SDM). C. The 30 nucleotide oligomer used to study protection of DNA by RPA against A3A.



Supplementary Figure S1. Proposed mechanism of UdgX and structure of UdgX complexed with DNA. A. The UdgX complex formation mechanism proposed by Ahn et al. 2019. The H178 residue coordinates the uracil in the catalytic pocket of UdgX initiating oxime formation and subsequent cleavage of the N-glycosidic bond. The H109 $N^{\delta 1}$ hydrogen is coordinated by E52 carboxylic acid, activating $N^{\varepsilon 2}$ for nucleophilic attack on the C1' carbon, resulting in a covalent bond between UdgX and the abasic site. B. Structural analysis of the H109 residue in UdgX (magenta; PDB 6AJO) covalently linked to the abasic site with uracil in an adjacent pocket (aqua). Orange sticks are the DNA backbone of the substrate, blue indicates nitrogen and red indicates oxygen atoms

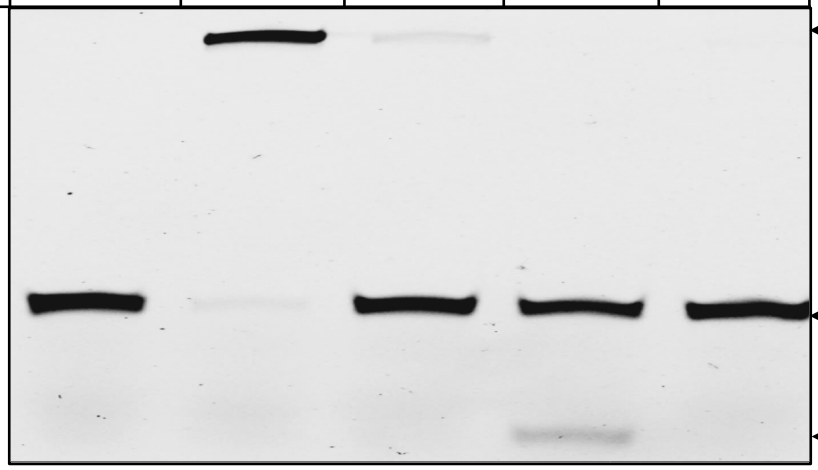


Supplementary Figure S2. UdgX expression constructs. A) The mCherry gene was deleted from pHis-mCherry-UdgX using NdeI restriction sites. B) A Shine-Delgarno sequence (red arrow) was introduced upstream of the FLAG-tag in the FLAG-HA-pcDNA3.1 vector by site-directed mutagenesis (SDM). The UdgX gene was amplified from pHis-UdgX then cloned into the FLAG-HA-pcDNA3.1 downstream of the HA tag. Polyglycine linkers separating UdgX from HA tag and HA tag from FLAG tag are indicated (black arrows).

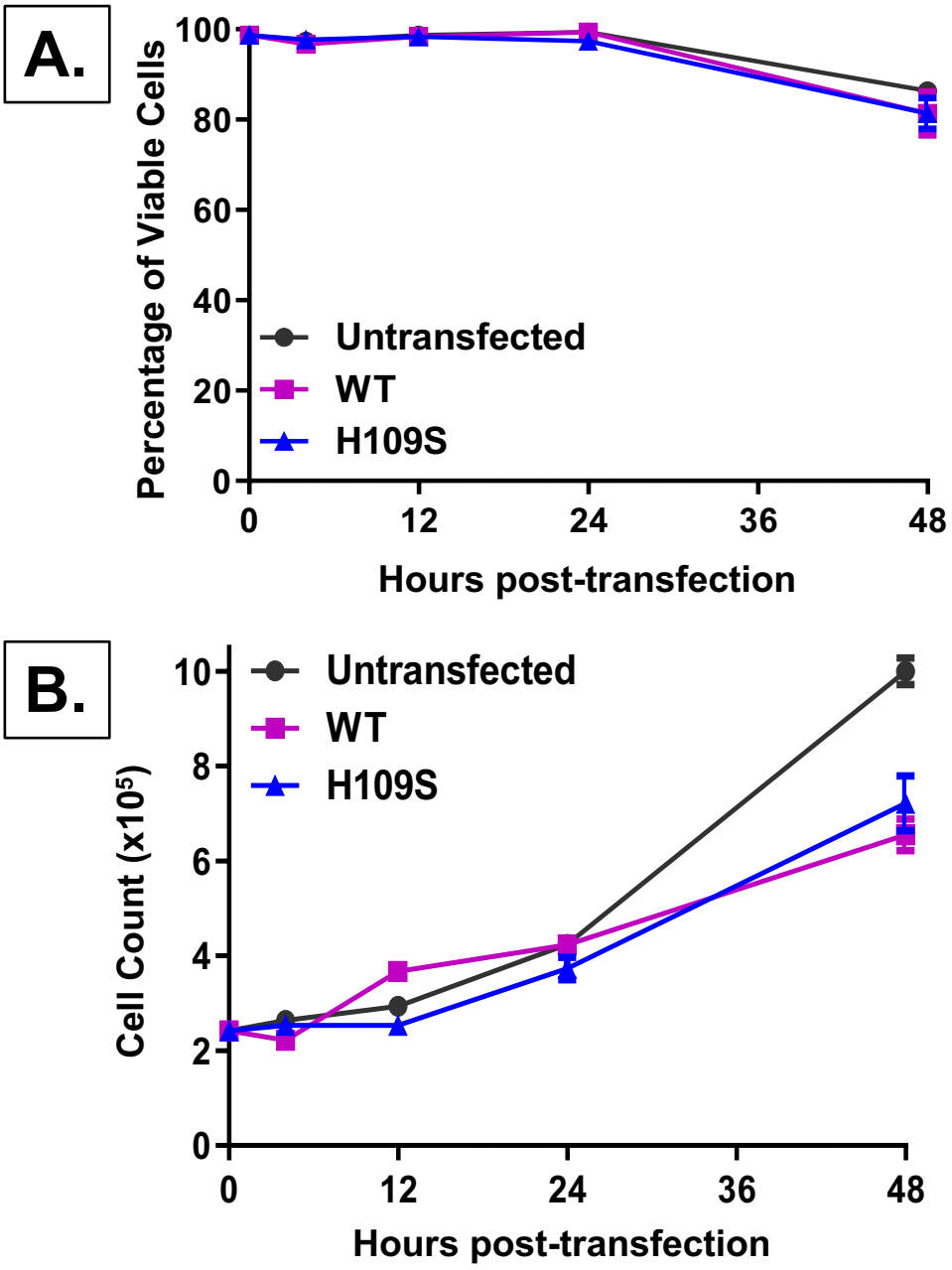


Supplementary Figure S3. Purified UdgX proteins. PolyHis-tagged and FLAG-tagged UdgX proteins were electrophoresed on an SDS-PAGE gel to judge the level of their purity. The approximate size of polyHis-UdgX (25 kDa) and FLAG-UdgX proteins (27 kDa) were compared to a precision plus protein standard.

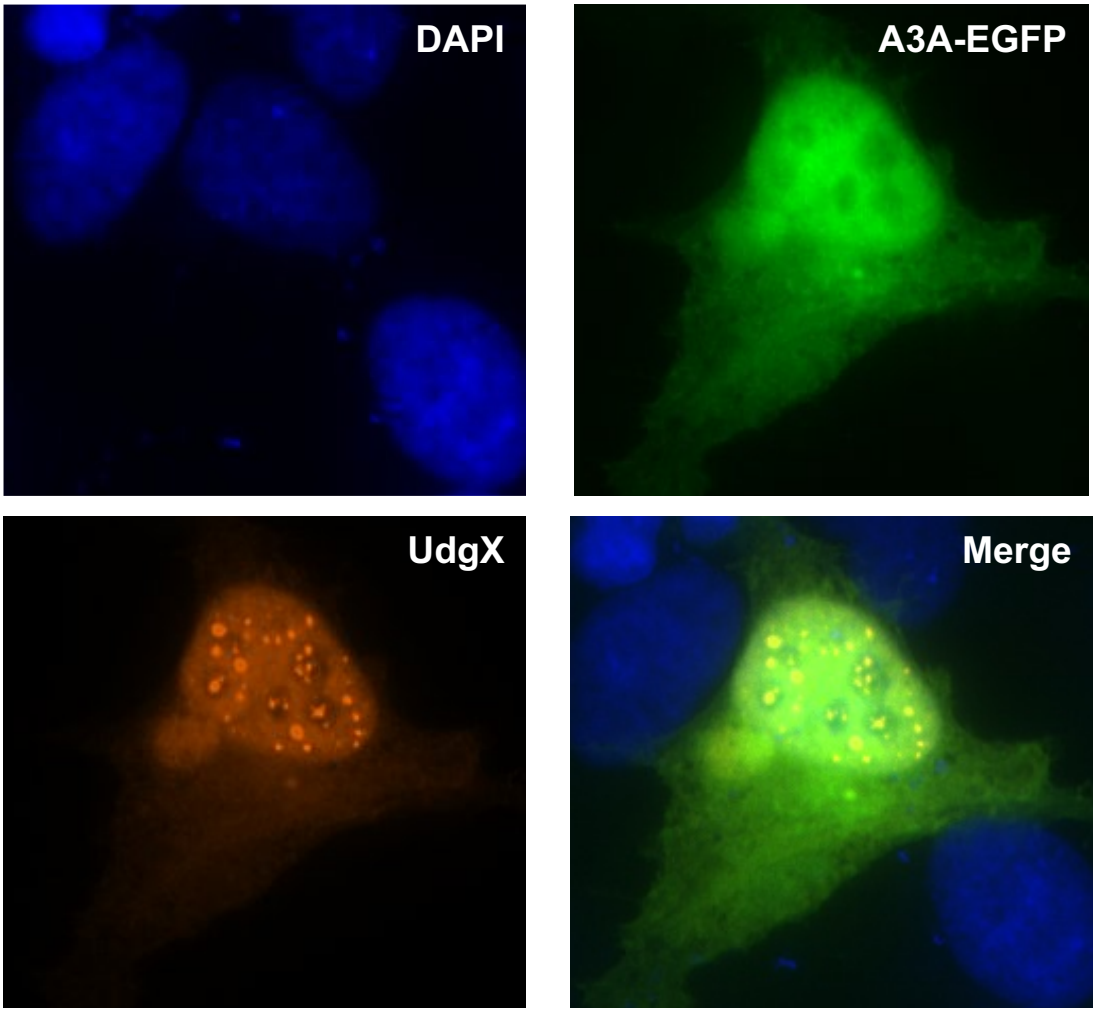
UdgX	-	WT	E52Q	H109S	H178A
Ugi	-	+	+	+	+
Percent Complex	0	97	6	0	0
Percent Product	0	0	0	20	0



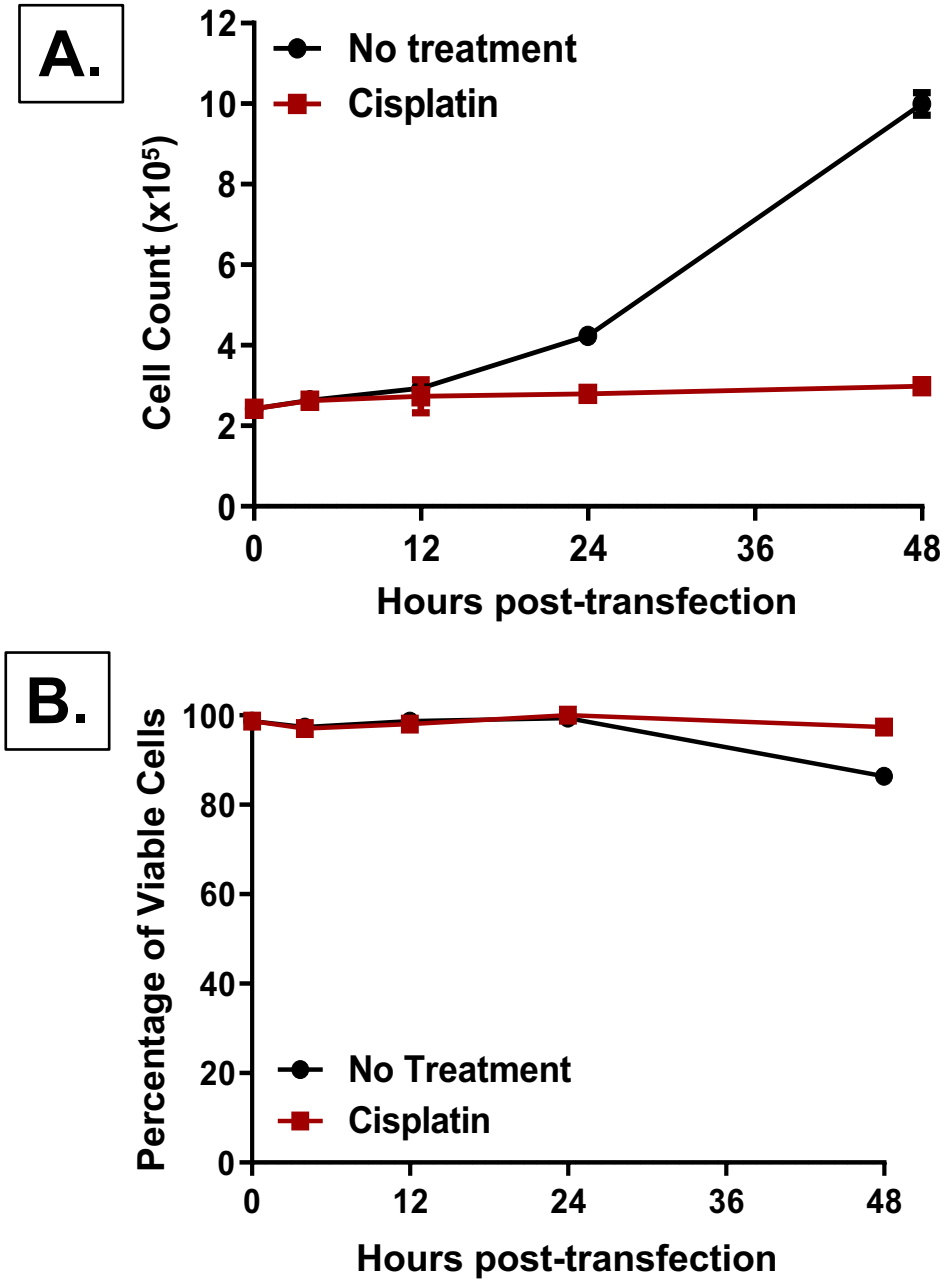
Supplementary Figure S4. UdgX mutant activity toward ssDNA. Homogeneous FLAG-UdgX WT or mutant proteins were reacted with a 6FAM-labeled oligomer containing a single uracil in UdgX optimized buffer. The samples were separated on a denaturing gel. H109S mutant weakly excises the uracil but does not form a covalent complex resulting in a faster migrating cleaved product. Other UdgX mutants (E52Q and H178A) were also purified and evaluated in parallel but will not be discussed here.



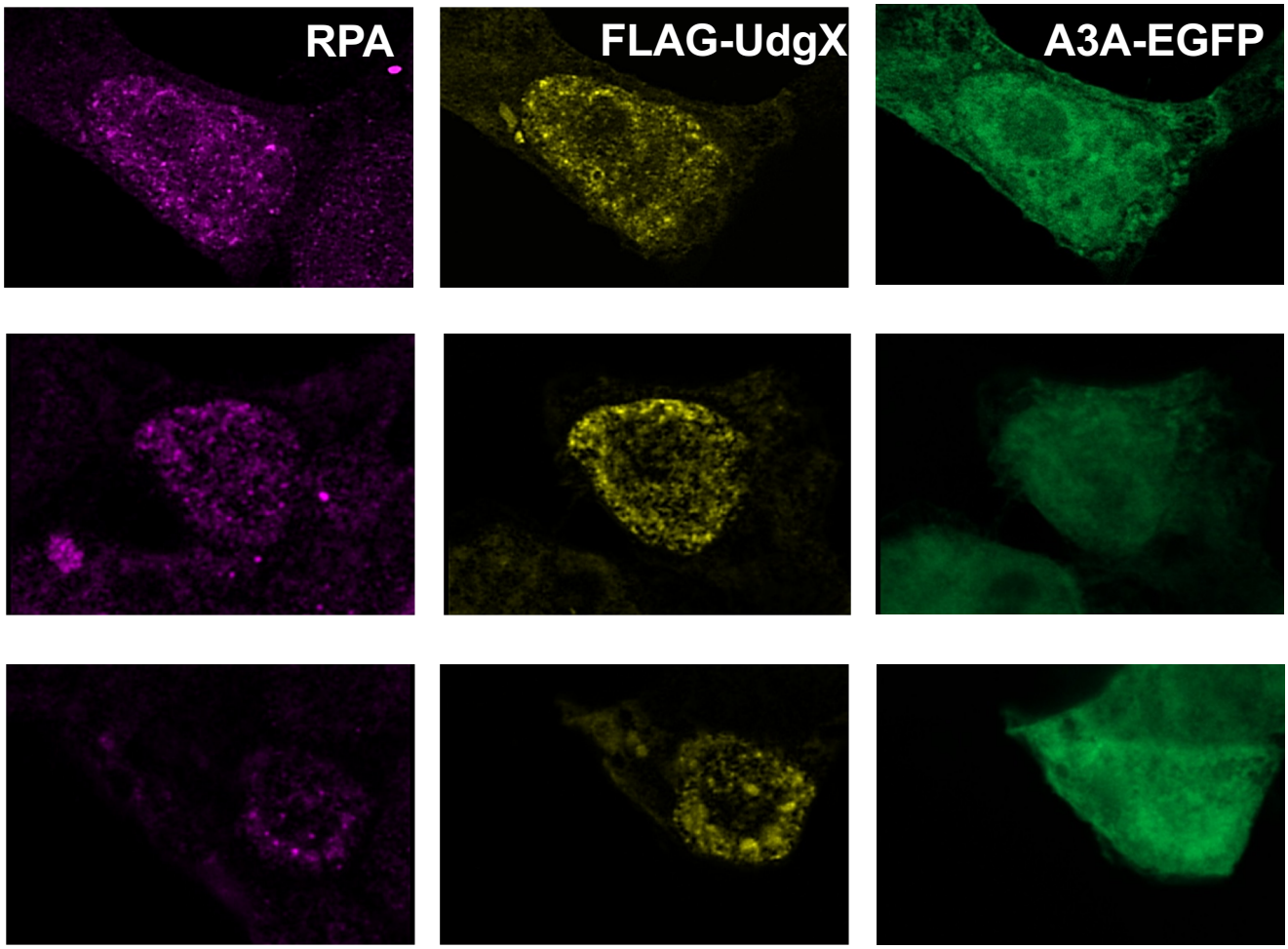
Supplementary Figure S5. UdgX transfection is not toxic to HEK293T. HEK293T cells were transfected with pFLAG-UdgX WT and the H109S mutant. At the different time points cells were evaluated for viability (A) and cell growth (B).



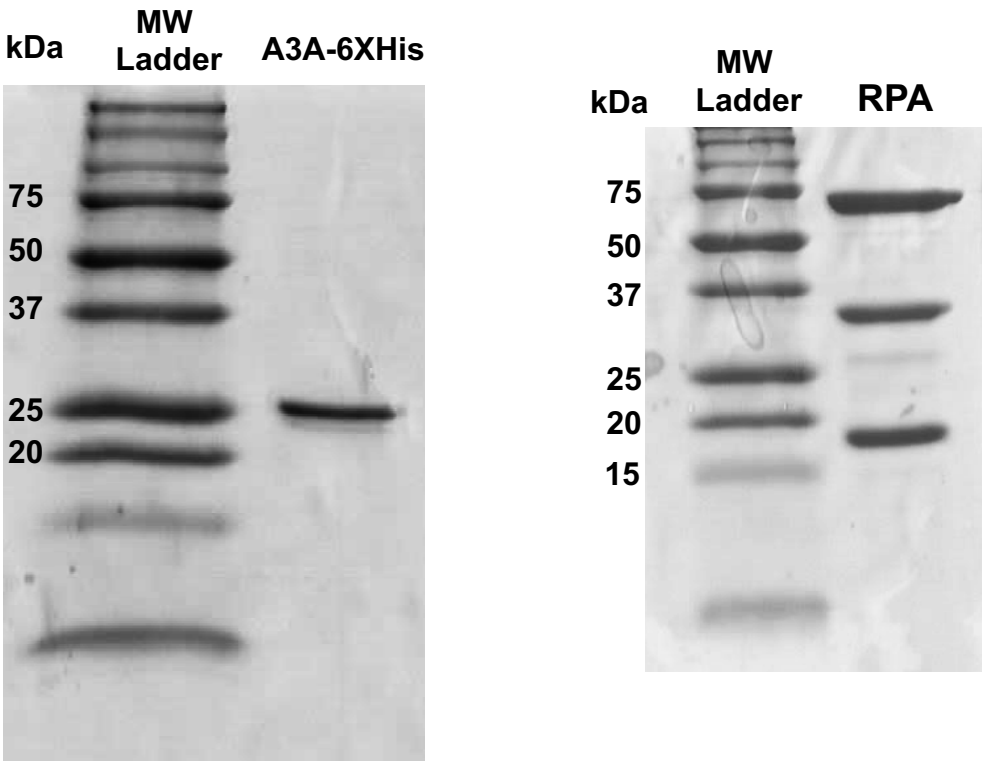
Supplementary Figure S6. Distribution of A3A and UdgX in cell nuclei. HTO-A3A-EGFP cells were induced for A3A-EGFP expression using doxycycline and transfected with pFLAG-UdgX. Anti-FLAG antibody was used to detect UdgX protein and DAPI stain was used to identify individual nuclei.



Supplementary Figure S7. Cisplatin treatment arrests HEK293T cell growth. HEK293T cells were treated with 5 μM cisplatin their growth growth (A) and viability (B) was monitored over 48 Hr.

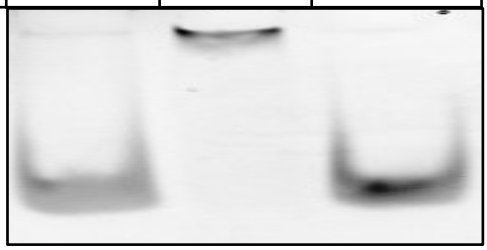


Supplementary Figure S8. High resolution images of UdgX and RPA foci. HEK293T cells were treated with cisplatin and transfected with pFLAG-UdgX and pA3A-EGFP. Cells were stained with anti-FLAG and anti-RPA antibodies then imaged using a confocal microscope.



Supplementary Figure S9. Purified A3A and RPA. Purified A3A and RPA were separated on SDS-PAGE gels and stained with Coomassie Brilliant Blue dye to judge their purity.

A3A Substrate (2 pmol)	**+**	**+**	**+**
RPA (4 pmol)	**-**	**+**	**+**
Unlabeled oligo	**-**	**-**	**+**



Supplementary Figure S10. Binding of RPA with 30-mer DNA. The fluorescently labeled DNA was incubated with a two-fold molar excess of RPA for 30 min. in a parallel reaction, the incubation with RPA was followed by the addition 100-fold excess of unlabeled 30-mer and the incubation was continued for an additional 15 minutes. The reactions and the unreacted 30-mer were electrophoresed on a non-denaturing gel.