Purification of Human ADAR Enables Evaluation of Oligo-Directed RNA Editing in a Cell Free Environment that Predicts In Vivo Activity

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Abstract

Adenosine Deaminase Acting on RNAs (ADARs) ability to revert G to A mutations in double stranded RNA (dsRNA) allows Korro to co-opt these endogenous enzymes using short, modified oligonucleotide known as CHORDs™ (Customized High-fidelity Oligonucleotides for RNA Deamination) to correct various disease targets or modulate protein function. To enable mechanistic studies of human ADAR1 and ADAR2 with our synthetic guide oligonucleotides, we report improved purification process for both isoforms of hADAR1 (p110 and p150) as well as hADAR2 in HEK293 cells. ADARs typically prefer to edit within an A-C mismatch sequence context, but the flanking sequences 5' and 3' to the edit site affect editing efficiency. We identified optimal nearest-neighbor pairings that enhance editing efficiency and revealed isoform-specific differences. Additionally, we demonstrated that the rank order of oligo-directed RNA editing in the cell-free assay corresponds to results in cell lines and *in vivo*. This robust purification procedure and the detailed kinetic characterization of guide RNAs will enable the next generation of chemical modifications for therapeutic RNA editing applications.

RNA Editing by ADARs

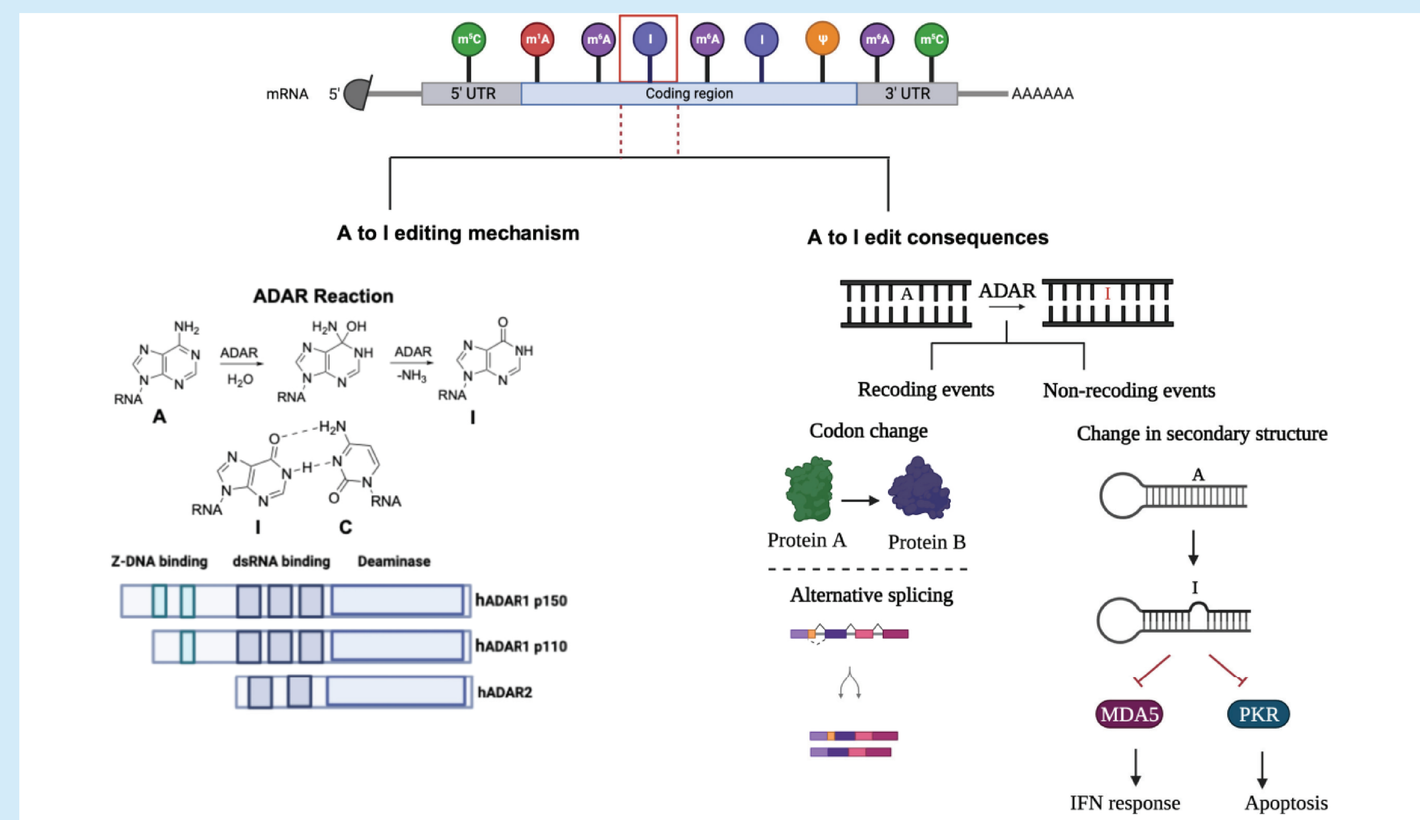
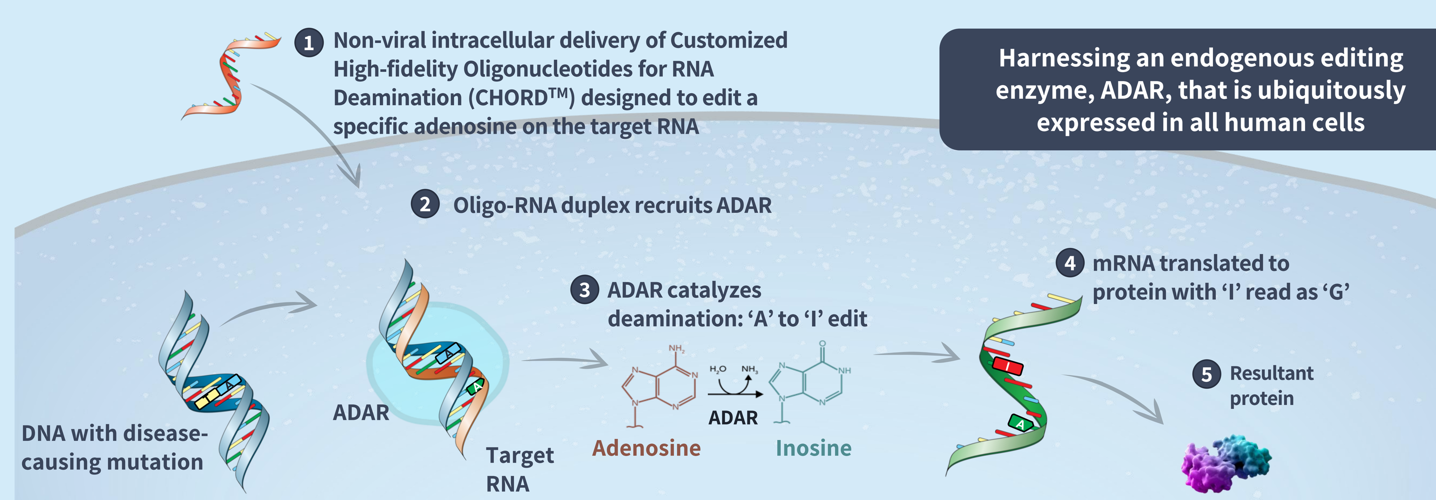


Figure 1: Adenosine deamination to inosine is a post-transcriptional modification that changes the base pairing capability of the nucleobase and is catalyzed by ADAR enzymes in double stranded RNA. A domain map of active ADAR isoforms is shown. Highlighted are consequences of A to I edit in RNA leading to recoding events and structural changes that lead to immunostimulatory responses.

Harnessing Endogenous ADAR with CHORDs



HEK293 Expression Enables Purification of all Human ADAR Isoforms

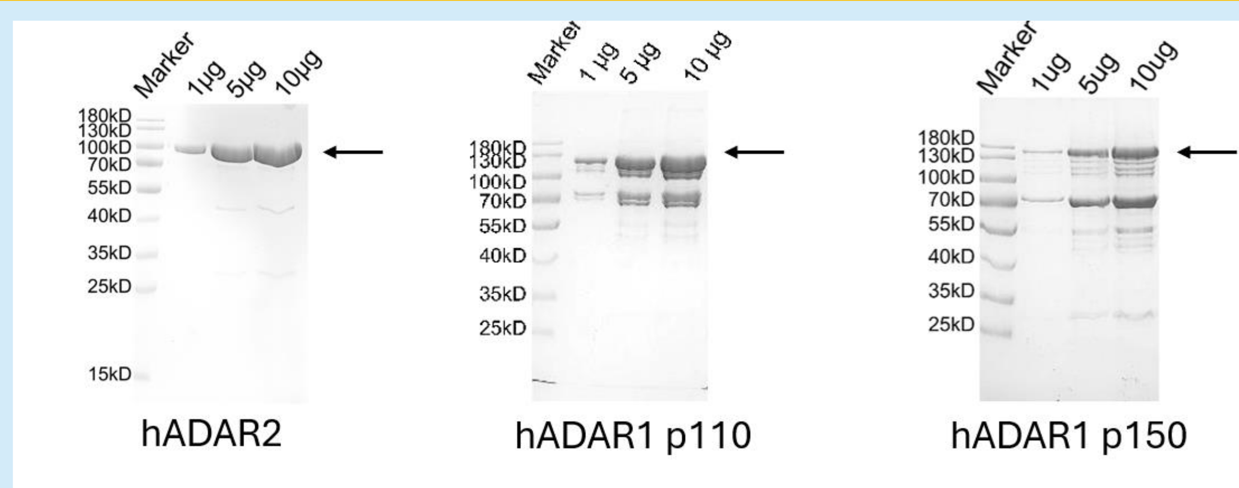
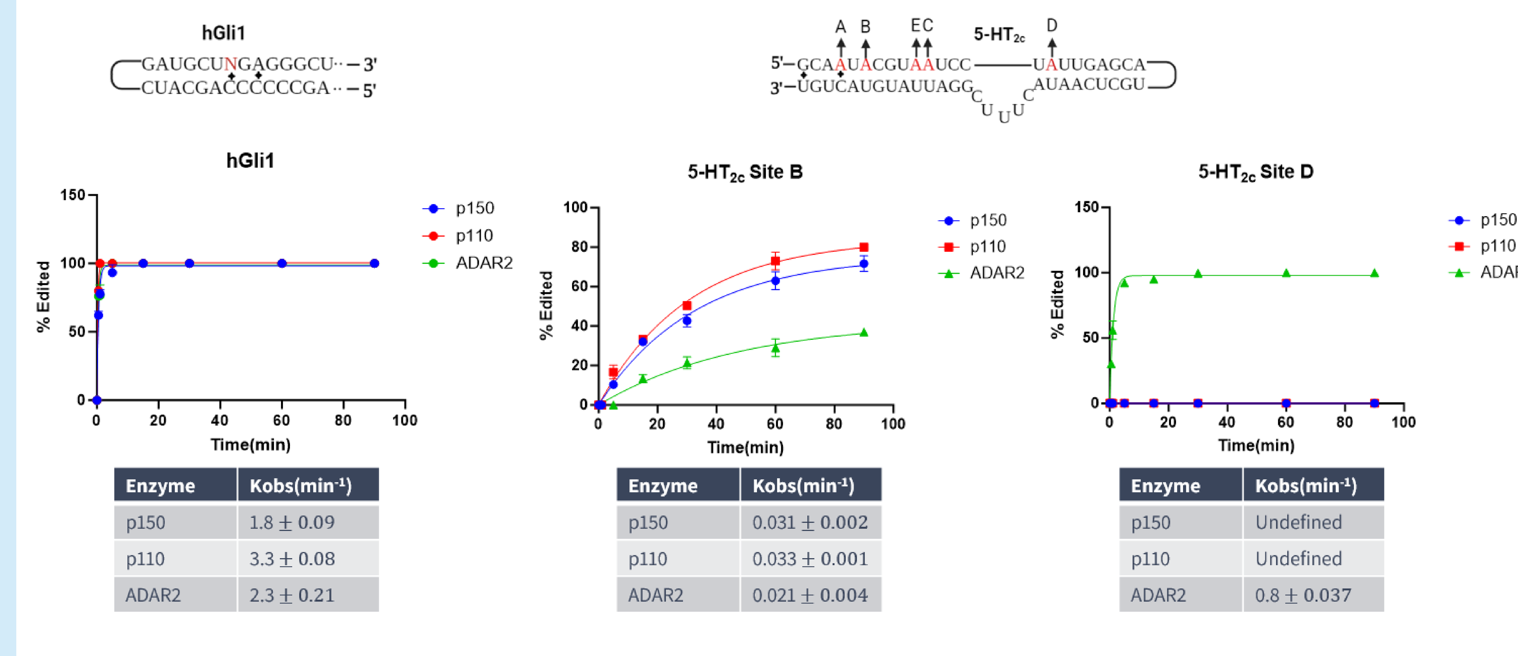


Figure 2: SDS-PAGE analysis of hADAR isoforms purified from HEK293 cells. A final protein yield of 3-5 mg was observed for all hADARs following 3L purifications. Longer ADAR isoforms contained a higher impurity profile and lower yields.

Natural Substrate Editing Comparison of hADAR Isoforms



hGli1:

Enzyme	Kobs(min⁻¹)
p150	1.8 ± 0.09
p110	3.3 ± 0.08
ADAR2	2.3 ± 0.21

5-HT2c Site B:

Enzyme	Kobs(min⁻¹)
p150	0.031 ± 0.002
p110	0.033 ± 0.001
ADAR2	0.021 ± 0.004

5-HT2c Site D:

Enzyme	Kobs(min⁻¹)
p150	Undefined
p110	Undefined
ADAR2	0.8 ± 0.037

Figure 3 : Validation of hADAR purification with natural substrates 5-HT$_{2c}$ and hGli1. Reactions were carried out in single turnover conditions with 50 nM enzyme and 5 nM substrate concentrations. As expected, all ADAR isoforms edited hGli1 and site-specific editing was observed for 5-HT$_{2c}$.

CHORD Activity *In Vitro* Translates to *In Vivo* Potency

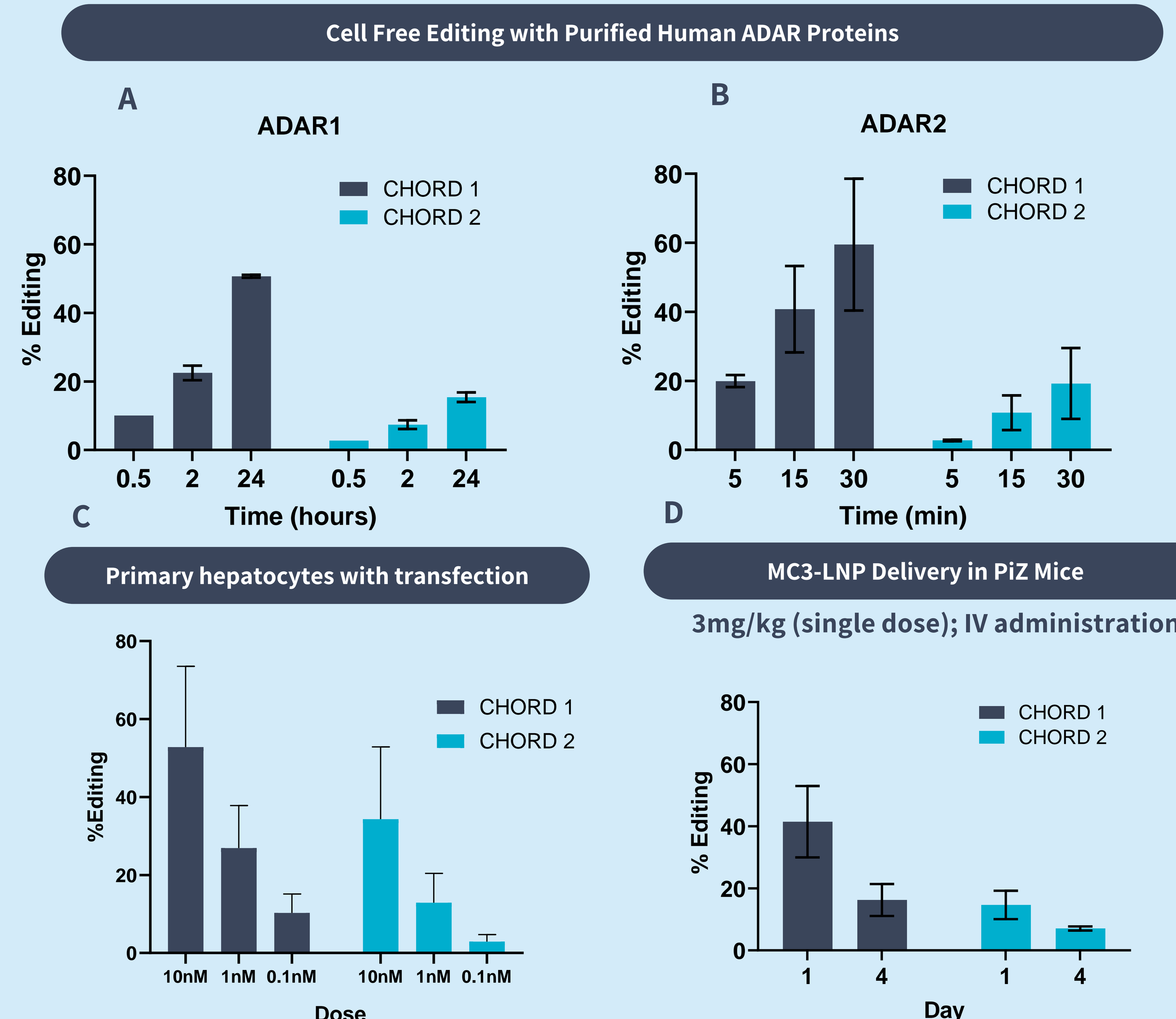


Figure 4 : The cell free assay is predictive of *in vivo* activity and differentiates chemical modifications that enhance intrinsic potency. **(A-B)** Cell free editing of a target with CHORDs and hADAR1 (0.5h, 2h, 24h) or hADAR2 (5min, 15min, 30min). **(C)** *In vitro* editing reflects the rank order of CHORDs from the cell free assay. **(D)** *In vivo* editing reflects the rank order of CHORDs observed in the cell free assay.

Target 5' and 3' Flanking Base Preference of ADARs

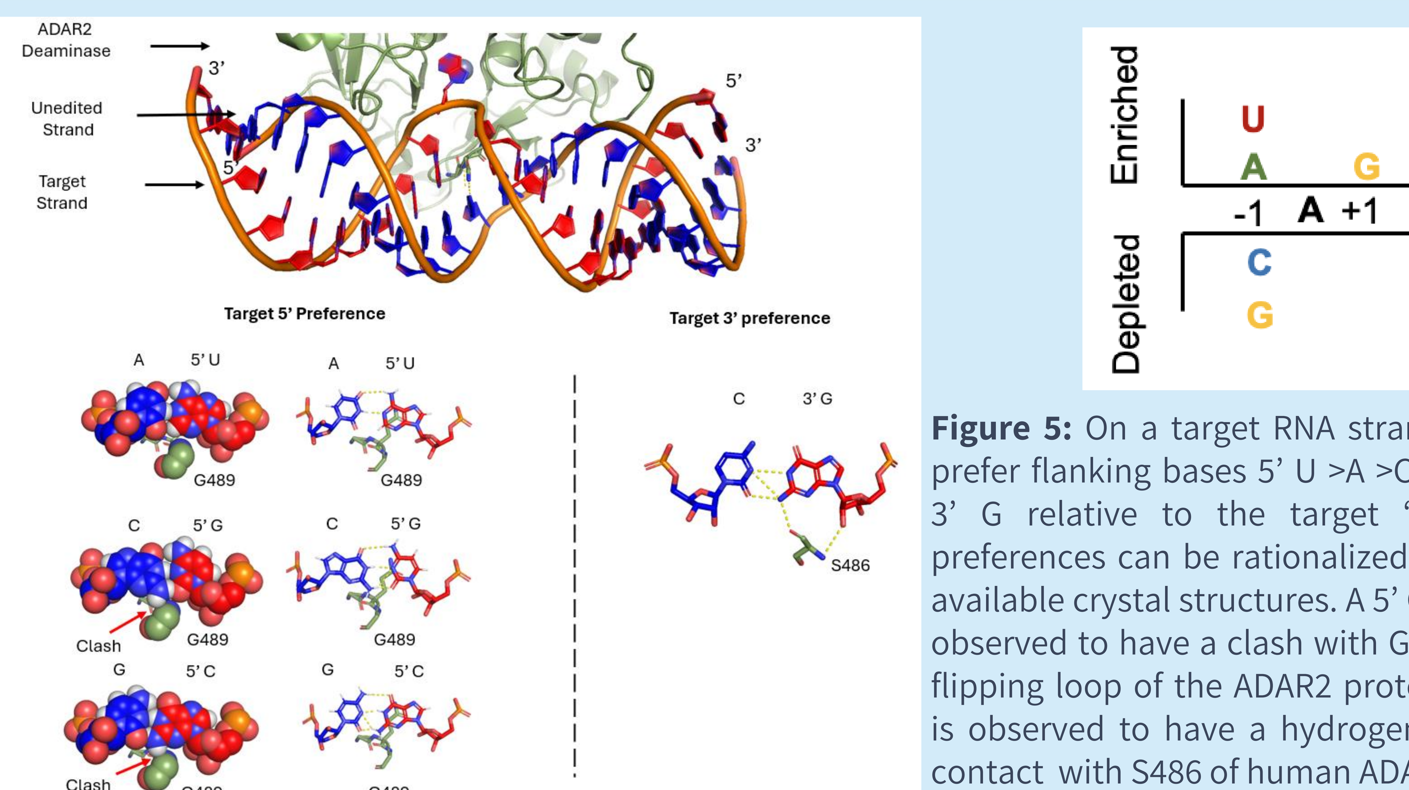


Figure 5: On a target RNA strand, ADARs prefer flanking bases 5' U >A >C >G and a 3' G relative to the target 'A'. These preferences can be rationalized based on available crystal structures. A 5' G or 5' C is observed to have a clash with G489 of the flipping loop of the ADAR2 protein. A 3' G is observed to have a hydrogen bonding contact with S486 of human ADAR2.

Cell Free Reaction Informs Optimal Nearest Neighbor Pairing in Trans

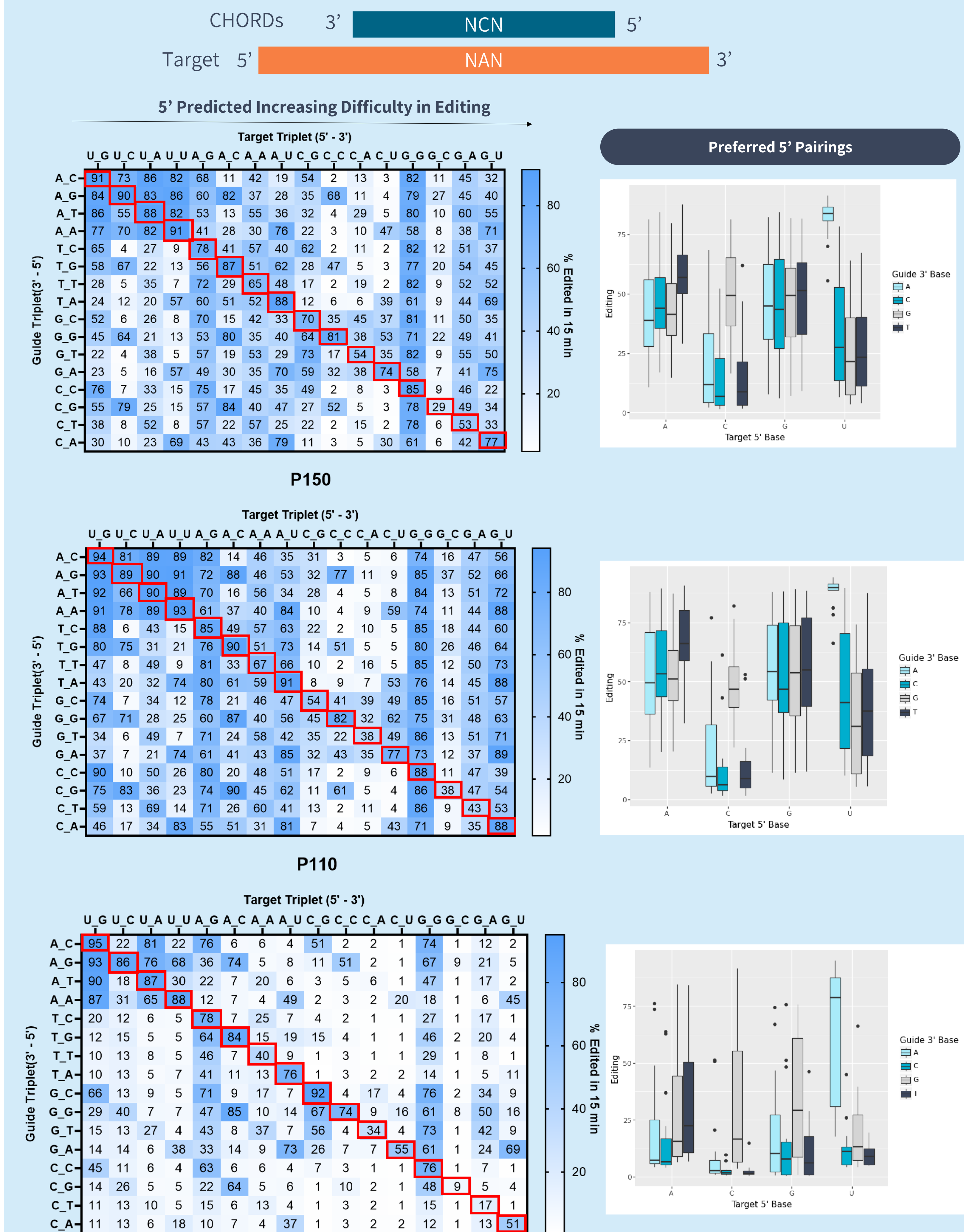


Figure 6 : Cell free editing informs nearest neighbor combinations that enable high editing. 11.2 nM of purified hADARs were reacted with 16 nearest neighbor combinations of a target RNA and CHORDs (2.25 nM duplex). Canonical pairings are highlighted in red. hADAR2 had the most stringent preferences for editing. 5' G sites were most difficult for all ADAR isoforms. Contrary to previous findings we find the 3' target nucleobase can strongly drive editing preferences.

Observations and Conclusions

- Human ADAR isoforms ADAR2, ADAR1 (p110), and ADAR1 (p150) can be expressed in HEK293 cells and purified in milligram quantities. Longer ADAR isoforms show a greater impurity profile and lower yield.
- Purified hADAR enzymes demonstrate robust activity with natural substrates and edit expected sites comparable to what is reported in the literature.
- The cell free editing system can be used to rank order chemical modifications that are beneficial for intrinsic potency. Good correlations can be observed between cell free editing and *in vivo* activity.
- Isoform-specific preferences for nearest neighbor pairings were determined using purified hADARs. Canonical pairings were preferred in most cases except in the context of 5' GAN where several non-canonical pairings demonstrated robust editing. All hADARs favored a 3'G adjacent to the edited A (see table below).

Target triplet preferences

Protein	5'	3'
p150	U>A>C>G	G
p110	U>A>C>G	G>U>C>A
ADAR2	U>A>G>C	G

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