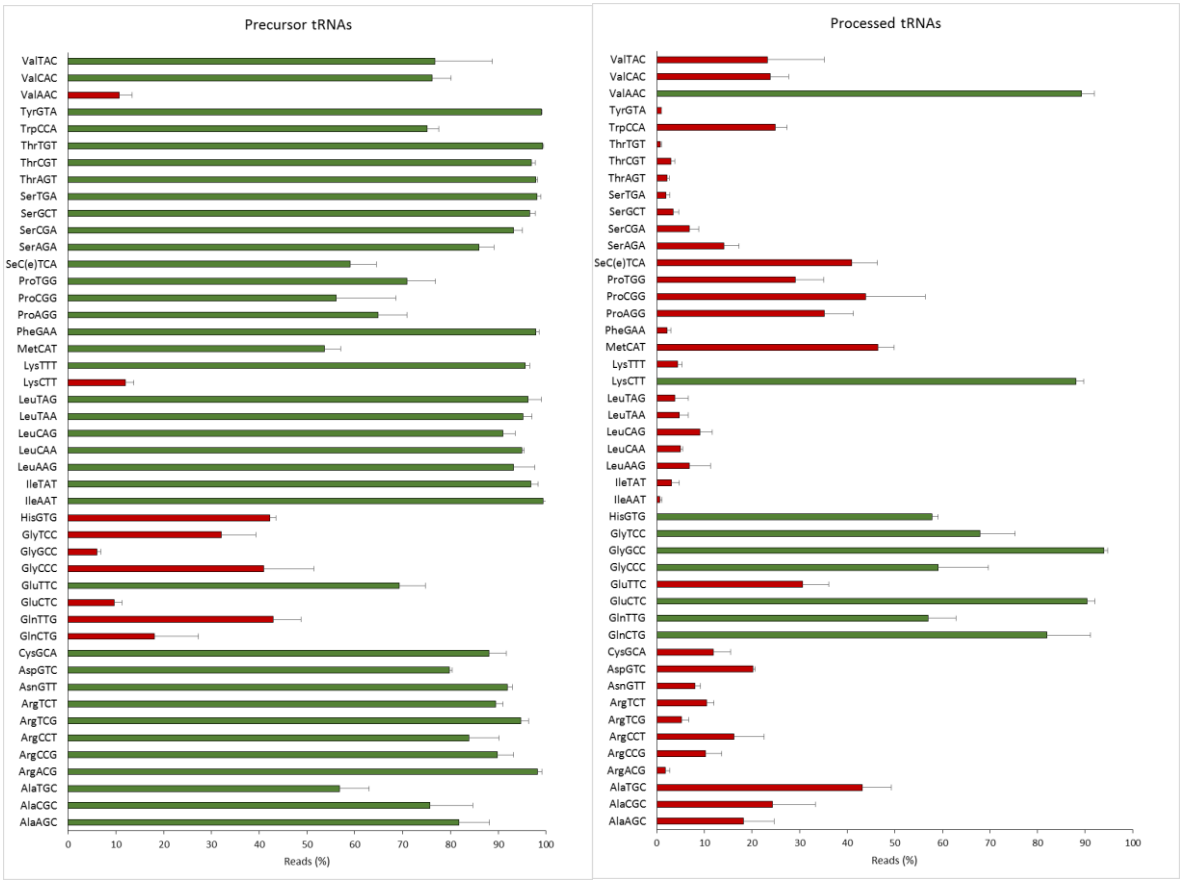
***Torres et al.*** **Inosine modifications in human tRNAs are incorporated at the precursor-tRNA level**

**Supplementary Figures and Table.**

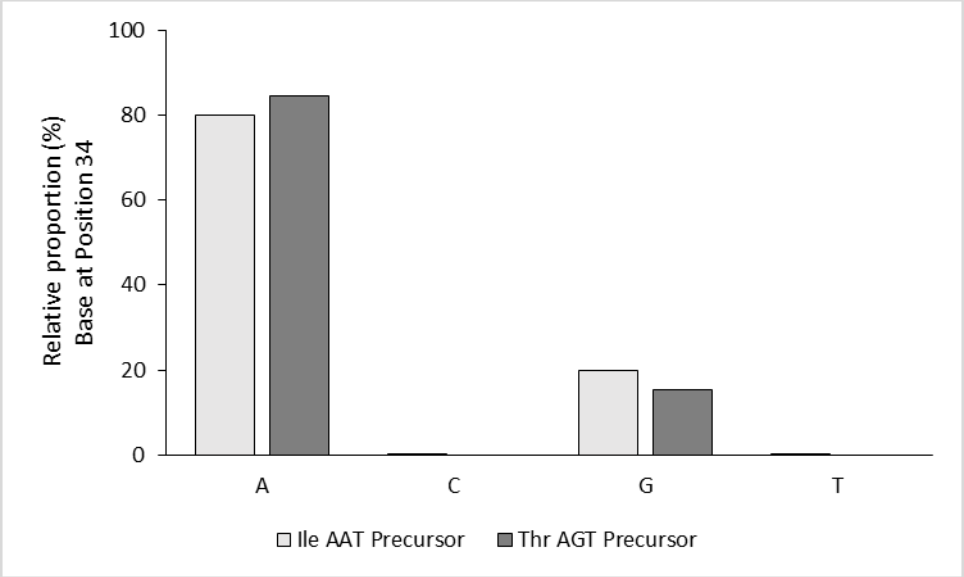
**Supplementary Table 1.** Oligonucleotides (DNA oligos) used in this study.

| Oligo | Sequence (5'-3') |
|---|---|
| pre-tRNA Val AAC oligo 1 | AGCTTAATACGACTCACTATAGGGTTGTTTCCGTAGTGT |
| pre-tRNA Val AAC oligo 2 | AGTGGTTATCACGTTCGCCTAACACGCGAAAGGTCCCTG |
| pre-tRNA Val AAC oligo 3 | GATCAAAACCAGGCGGAAACAAGTGGTTACCCTTCTTACATGTG |
| pre-tRNA Val AAC oligo 4 | ATAACCACTACACTACGGAAACAACCCTATAGTGAGTCGTATTA |
| pre-tRNA Val AAC oligo 5 | TTTTGATCCAGGGACCTTTCGCGTGTTAGGCGAACGTG |
| pre-tRNA Val AAC oligo 6 | GATCCACATGTAAGAAGGGTAACCACTTGTTTCCGCCTGG |
| Forward primer HsADAT2 In-Fusion pPEU17 (ADAT2-NtermCherry) | AAGTTCTGTTTCAGGGCCCGGAGGCGAAGGCGGCACCCA |
| Reverse primer HsADAT2 In-FusionpPEU17 (ADAT2-NtermCherry) | ATGGTCTAGAAAGCTTTAAGATTTCTGACATTCCTTTTTCCGAAC |
| Forward primer HsADAT2 In-Fusion pPEU19 (ADAT2-CtermCherry) | AGGAGATATACCATGGAGGCGAAGGCGGCACCCAA |
| Reverse primer HsADAT2 In-Fusion pPEU19 (ADAT2-CtermCherry) | CTTCCAGACCGCTTGAAGATTTCTGACATTCCTTTTTCCGAAC |
| Forward primer HsADAT3 In-Fusion pPEU17 (ADAT3-NtermCherry) | AAGTTCTGTTTCAGGGCCCGATCCTCTGCTCCCGTCTCTGTCT |
| Reverse primer HsADAT3 In-Fusion pPEU17 (ADAT3-NtermCherry) | ATGGTCTAGAAAGCTTTACGTGTCGGGGTCCAGCCAGCG |
| Forward primer HsADAT3 In-Fusion pPEU19 (ADAT3-CtermCherry) | AGGAGATATACCATGATCCTCTGCTCCCGTCTCTGTCT |
| Reverse primer HsADAT3 In-Fusion pPEU19 (ADAT3-CtermCherry) | CTTCCAGACCGCTTGACGTGTCGGGGTCCAGCCAGCG |
| Forward primer HsADAT2 pEGFP-N1 (ADAT2-CtermGFP) | TTATAAGCTTATGGAGGCGAAGGCGGCA |
| Reverse primer HsADAT2 pEGFP-N1 (ADAT2-CtermGFP) | AACTGGTACCCAAGATTTCTGACATTCCTTTTTCCG |
| Forward primer HsADAT2 pEGFP-C1 (ADAT2-NtermGFP) | TTATAAGCTTTGGAGGCGAAGGCGGCAC |
| Reverse primer HsADAT2 pEGFP-C1 (ADAT2-NtermGFP) | TATTGGTACCTCAAGATTTCTGACATTCCTTTTTCC |
| Forward primer HsADAT3 pEGFP-N1 (ADAT3-CtermGFP) | ATATAAGCTTATGATCCTCTGCTCCCGTCT |
| Reverse primer HsADAT3 pEGFP-N1 (ADAT3-CtermGFP) | TATAGGTACCGTGTCGGGGTCCAGCC |
| Forward primer HsADAT3 pEGFP-C1 (ADAT3-NtermGFP) | ATATAAGCTTTGATCCTCTGCTCCCGTCTC |
| Reverse primer HsADAT3 pEGFP-C1 (ADAT3-NtermGFP) | TATAGGTACCTACGTGTCGGGGTCCAG |
| Forward primer RT-qPCR HsADAT2 | AAGGCTGAGATTGGAAGCTG |
| Reverse primer RT-qPCR HsADAT2 | AGCAATTCACCATGAGCTGT |
| tRNA Ala AGC oligo 1 | AGCTTAATACGACTCACTATAGGGGGTGTGGCT |
| tRNA Ala AGC oligo 2 | CAGTGGTAGAGCGCGTGCTTAGCATGCACGAG |
| tRNA Ala AGC oligo 3 | GCCCCGGGTTCAATCCCCGGCACCTCCACCAGGG |
| tRNA Ala AGC oligo 4 | CGCTCTACCACTGAGCCACACCCCCTATAGTGAGTCGTATTA |
| tRNA Ala AGC oligo 5 | TTGAACCCGGGGCCTCGTGCATGCTAAGCACG |
| tRNA Ala AGC oligo 6 | GATCCCCTGGTGGAGGTGCCGGGGA |

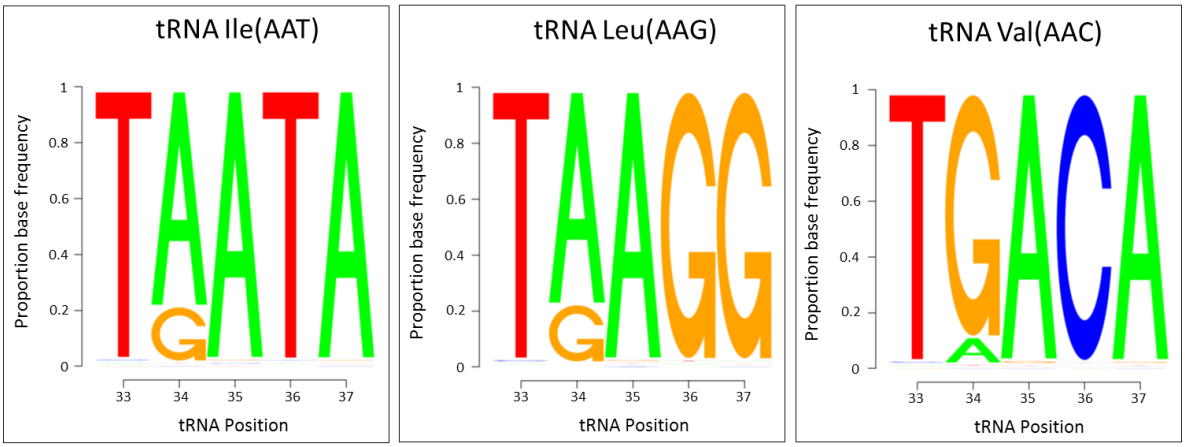
**Supplementary Figure 1**. Proportion (%) of tRNA reads corresponding to the Precursor tRNA group (left panel) or the Processed tRNA group (right panel) for every tRNA type detected by RNAseq in our study. Replicates correspond to 4 independent RNAseq experiments.



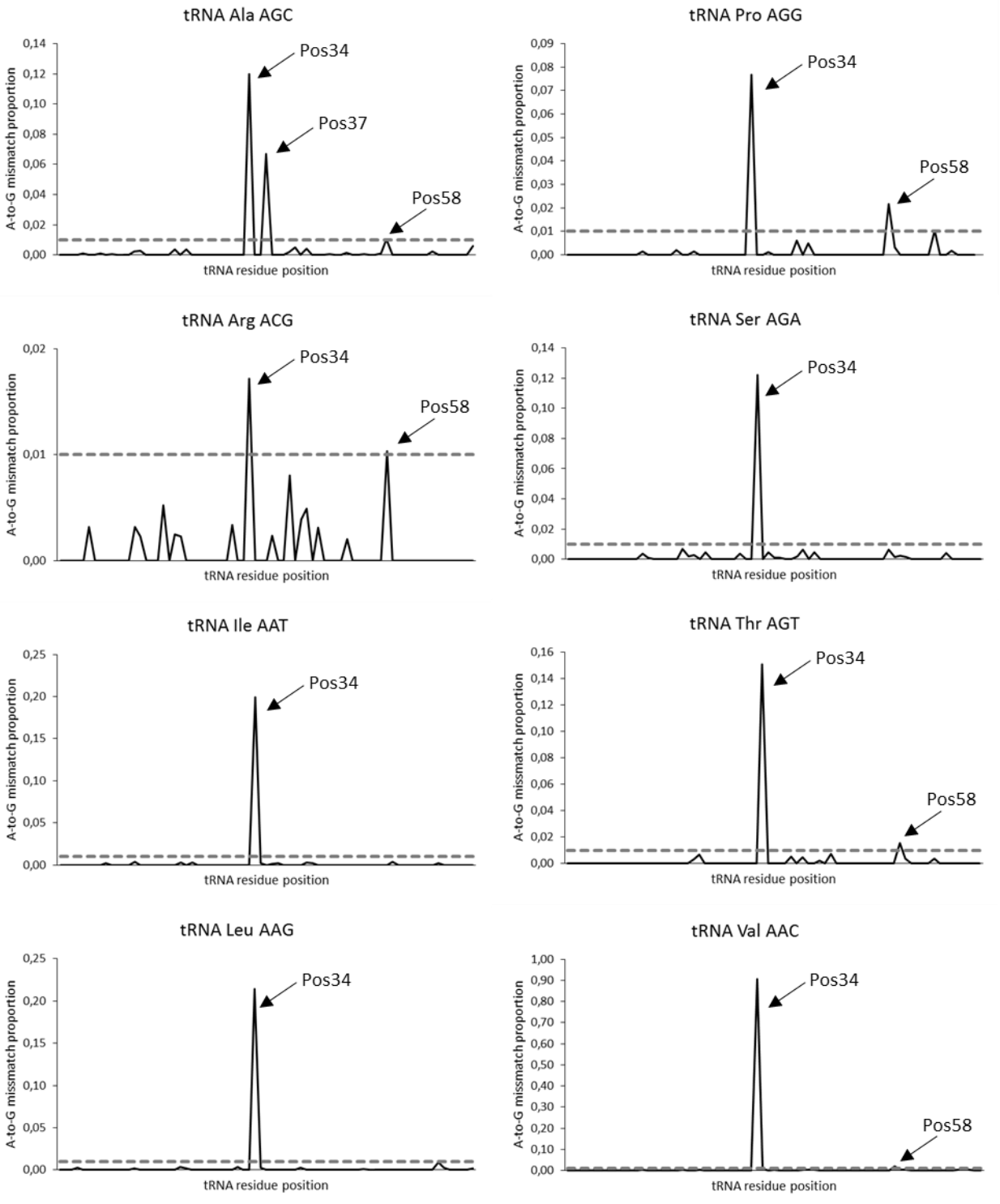
**Supplementary Figure 2.** Proportion (%) of bases found at position 34 for tRNA Ile AAT and tRNA Thr AGT within the "Precursor" tRNA group. In both cases inosine 34 (read as a G34) could be detected in the "Precursor" tRNA group. The "Processed" tRNA group was not analyzed for these tRNAs because of low read counts. We obtained less than 30 reads for "Processed" tRNA Ile AGT and less than 10 reads for "Processed" tRNA Thr AGT.



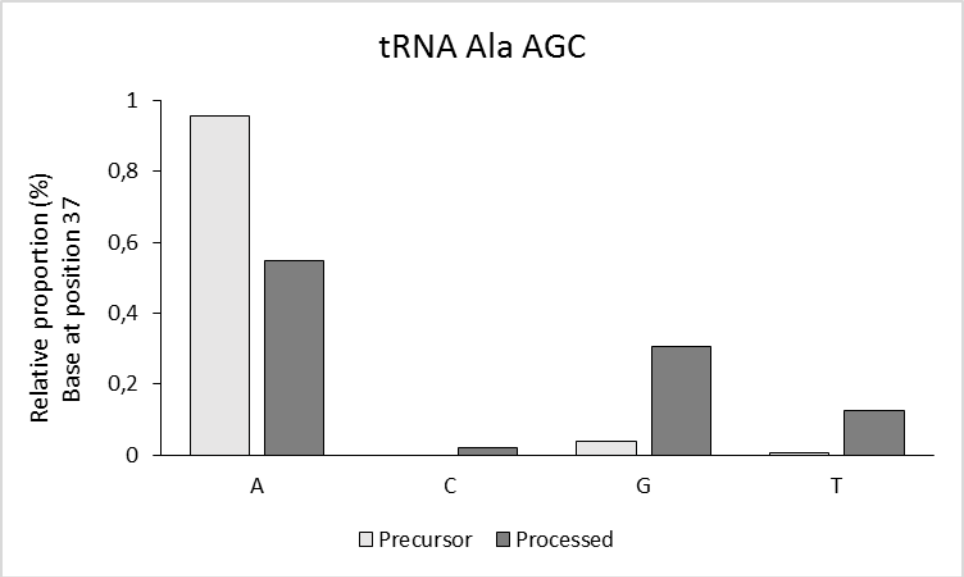
**Supplementary Figure 3.** The SeqLogo package version 1.24.0 was used to generate sequence logos showing nucleotide proportions around anticodon+1 position for tRNAs Ile(AAT), Leu(AAG) and Val(AAC). Shown are the results for base calls considering reads from the "Precursor tRNA" and "Processed precursor tRNA" groups together, for each tRNA type. While A-to-I deamination is observed at position A34 (detected as G34), no deamination is detected at adenosines in position 35 (A35) in these tRNAs.



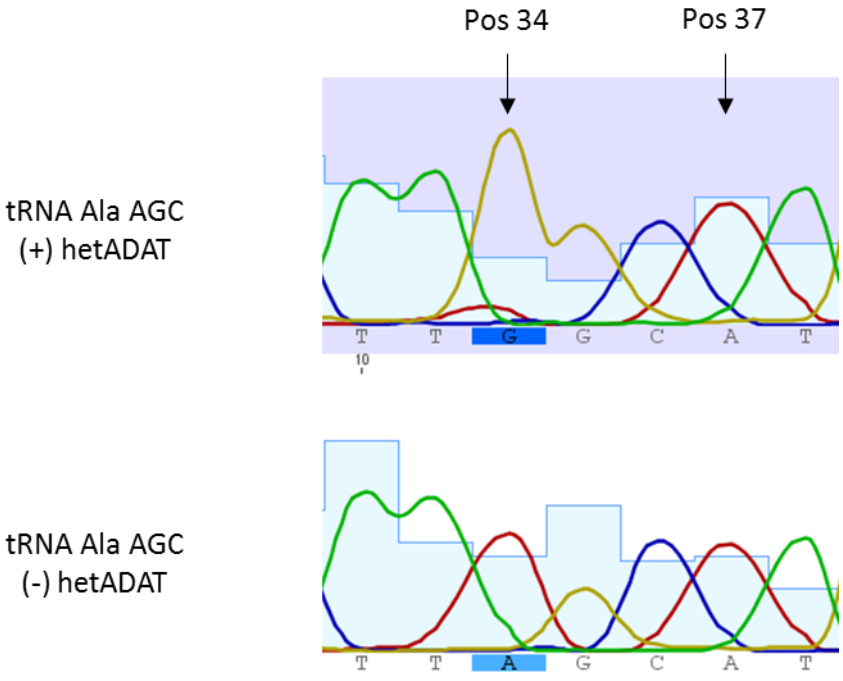

**Supplementary Figure 4** (see next page). A-to-G mismatch proportion observed by RNAseq at residues throughout the whole sequence of the tRNA for all human ANN tRNAs. Background/noise levels were defined as the signal below 1% of the A-to-G mismatch error rate and is represented in the graphs as a grey dashed line. Residues showing an A-to-G mismatch proportion higher than 1% are indicated. Pos34: I34; Pos37: I37 (will be further modified to $m^1$I37); Pos58: likely corresponds to $m^1$A ($m^1$A detected as a "G" when sequenced has been reported previously (30, 41, 42)). Note that each graph is depicted using different scales at the y-axes showing the strong sequencing biases towards detection of the A-to-G mismatch proportion in different tRNA types.


tRNA Ala AGC
A-to-G mismatch proportion
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
Pos34
Pos37
Pos58
tRNA residue position
tRNA Pro AGG
A-to-G missmatch proportion
0,09
0,08
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0,00
Pos34
Pos58
tRNA residue position
tRNA Arg ACG
A-to-G mismatch proportion
0,02
0,01
0,00
Pos34
Pos58
tRNA residue position
tRNA Ser AGA
A-to-G missmatch proportion
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
Pos34
tRNA residue position
tRNA Ile AAT
A-to-G mismatch proportion
0,25
0,20
0,15
0,10
0,05
0,00
Pos34
tRNA residue position
tRNA Thr AGT
A-to-G missmatch proportion
0,16
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
Pos34
Pos58
tRNA residue position
tRNA Leu AAG
A-to-G missmatch proportion
0,25
0,20
0,15
0,10
0,05
0,00
Pos34
tRNA residue position
tRNA Val AAC
A-to-G missmatch proportion
1,00
0,90
0,80
0,70
0,60
0,50
0,40
0,30
0,20
0,10
0,00
Pos34
Pos58
tRNA residue position

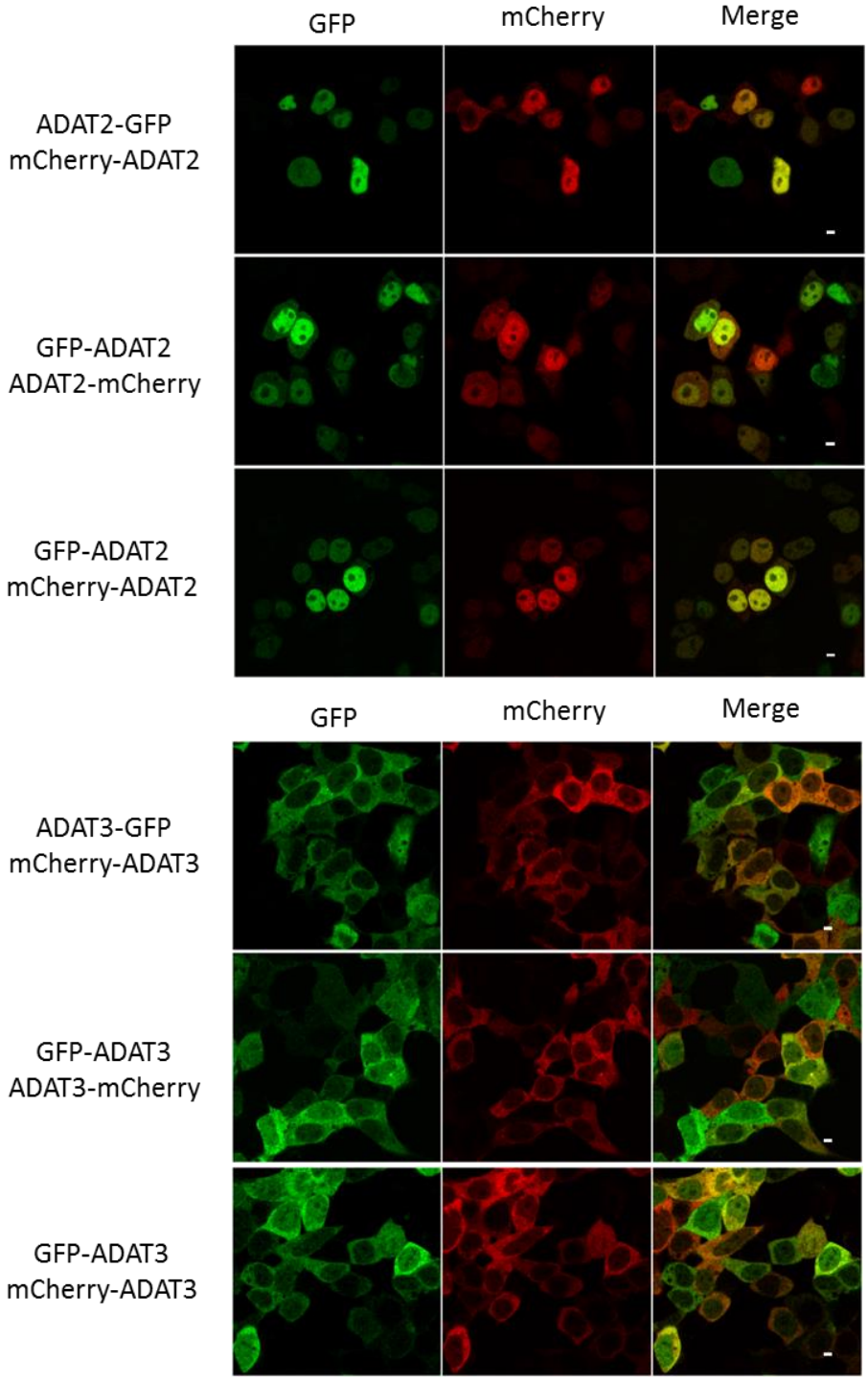
**Supplementary Figure 5.** Proportion (%) of bases found at position 37 for tRNA Ala AGC. Only A37 (unmodified A37) and G37 (I37) could be detected in the Precursor tRNA group. In the Processed tRNA group higher levels of G37 (I37) and T37 ($m^1$I37) could be detected.

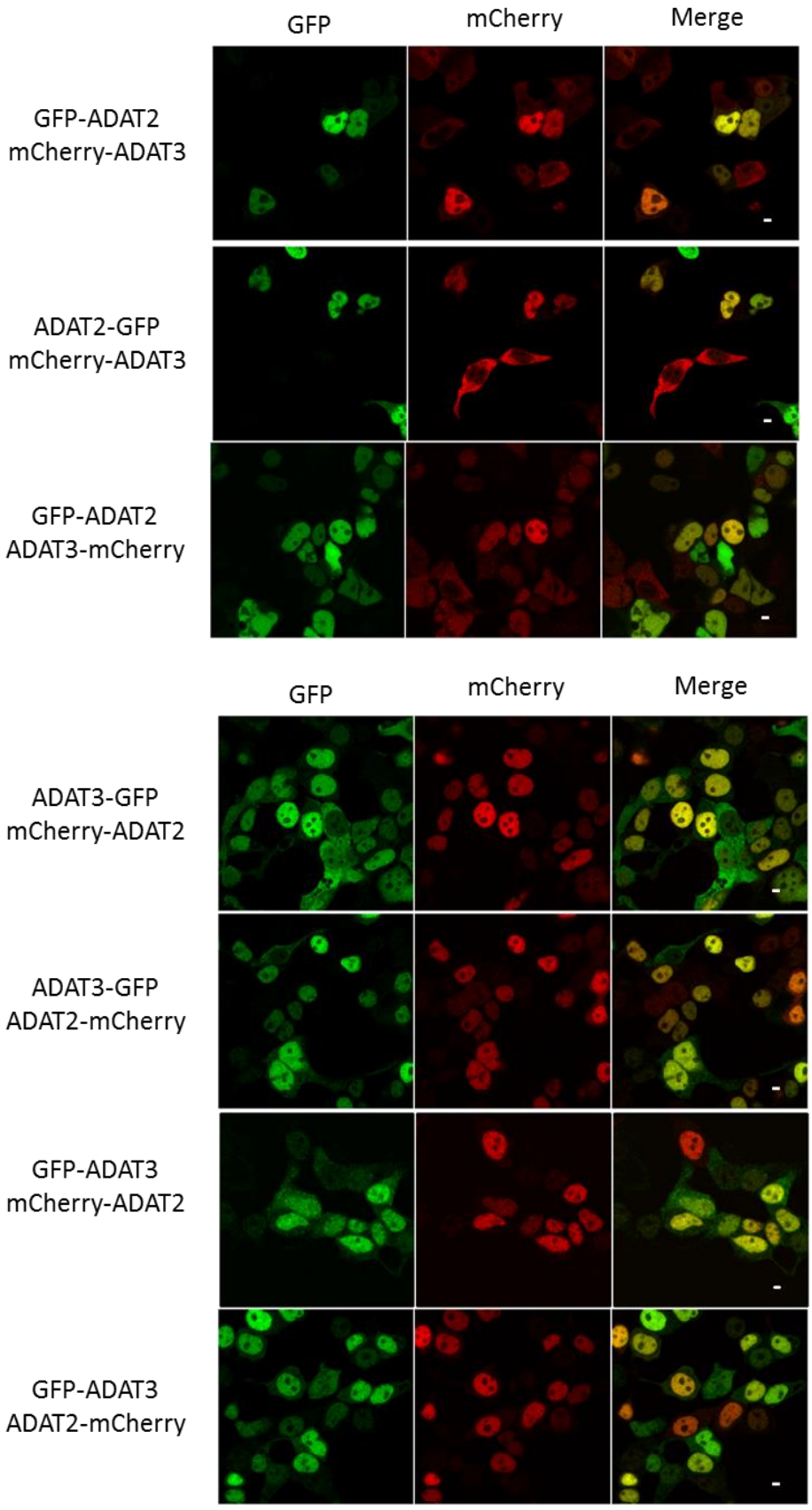



**Supplementary Figure 6.** Chromatogram obtained after sequencing of the anticodon loop of *in vitro* transcribed $tRNA^{Ala}_{(AGC)}$ incubated ((+) hetADAT) or not ((-) hetADAT) with purified human hetADAT. The experiment was carried out exactly as described for pre-$tRNA^{Val}_{(AAC)}$ in **Figure 2**. As expected, A37 was not deaminated by hetADAT.



**Supplementary Figure 7.** Live imaging confocal microscopy of HEK293T cells following co-expression of GFP- or mCherry-tagged HsADAT2 and HsADAT3 proteins. Scale bar corresponds to 5 µm. Shown are the results for all the combinations of C-term tagged or N-term tagged ADAT proteins not shown in **Figure 3**.




GFP
mCherry
Merge
GFP-ADAT2
mCherry-ADAT3
ADAT2-GFP
mCherry-ADAT3
GFP-ADAT2
ADAT3-mCherry
GFP
mCherry
Merge
ADAT3-GFP
mCherry-ADAT2
ADAT3-GFP
ADAT2-mCherry
GFP-ADAT3
mCherry-ADAT2
GFP-ADAT3
ADAT2-mCherry

**Supplementary Figure 8.** Non-normalized proportion (%) of I34 observed in HEK293T shCV cells and HEK293T shADAT2 cells for all human ANN tRNAs. Figure corresponds to the non-normalized values of **Figure 4B**.