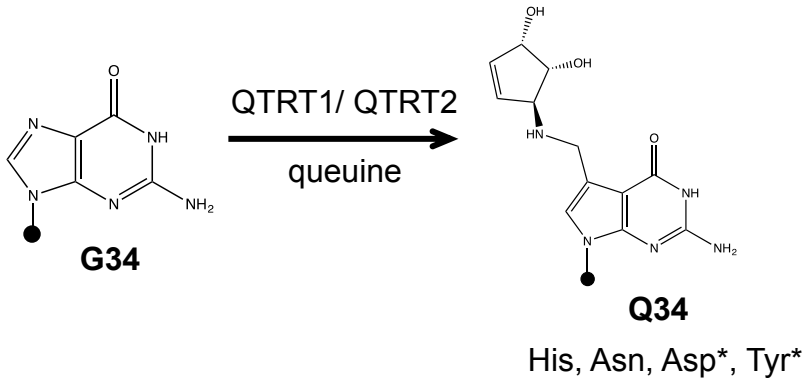


Fig. S1. Queuosine (Q) tRNA modification in four tRNAs. Schematic diagram of G34 to Q34 modifications in the wobble anticodon position of $tRNA^{His/Asn/Asp/Tyr}$. QTRT1 and QTRT2 (formerly QTRTD1) are encoded in the human genome. Black dots represent the body of these four tRNAs; the QTRT1/QTRT2 complex introduces Q modification in the place of G. Q34 residues in $tRNA^{Asp}$ and $tRNA^{Tyr}$ are further glycosylated at the cis-diol of the modified base, indicated by asterisks. Our quantitation method measures $tRNA^{His}$ and $tRNA^{Asn}$, but not $tRNA^{Asp}$ and $tRNA^{Tyr}$ because of their glycosylation.

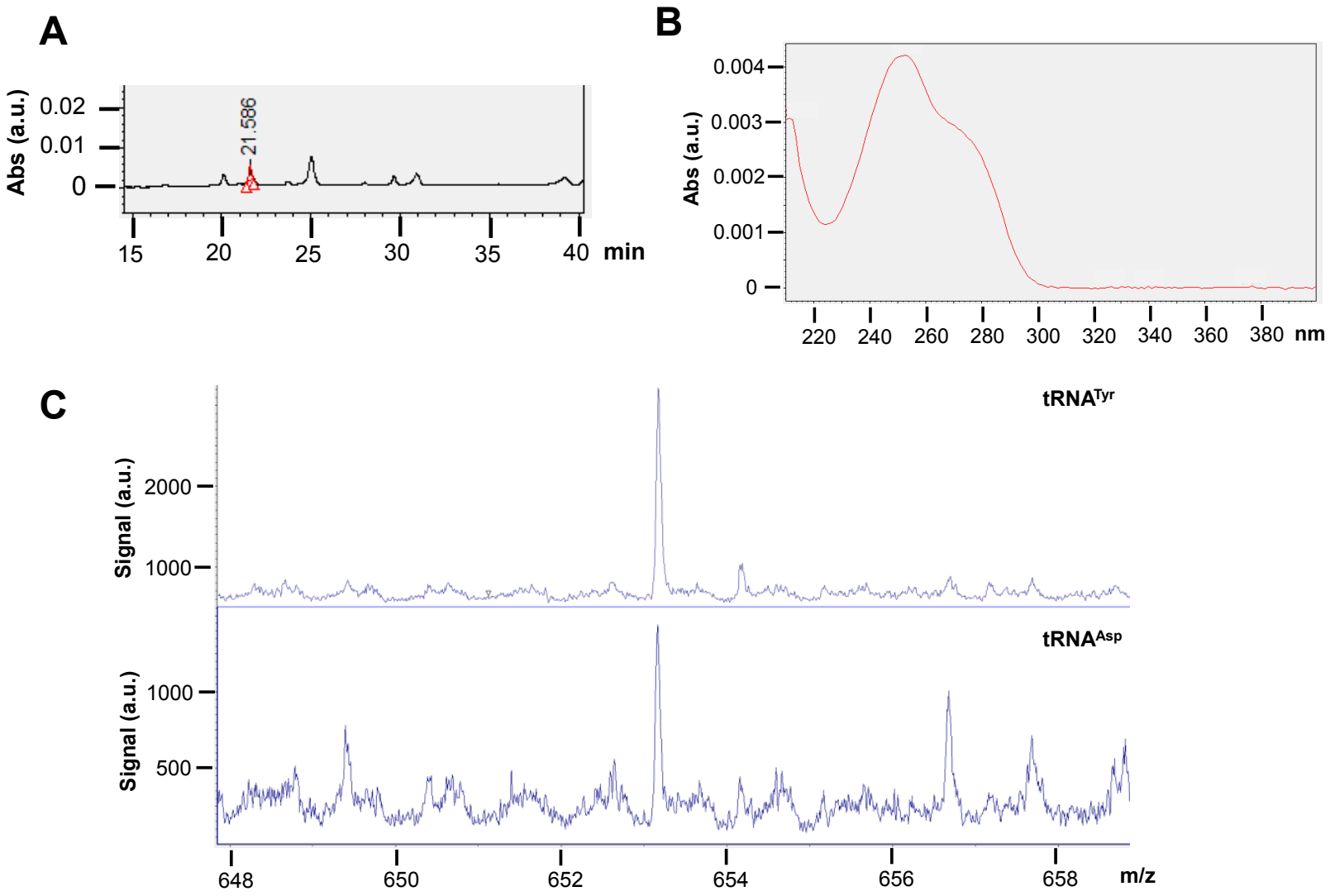


Fig. S2: Identification of glycosylated Q-tRNA modifications by HPLC and MS. (A) HPLC chromatogram of $tRNA^{Tyr}$ digested to mononucleotide 5' phosphate. (B) Absorbance of the peak at 21.586 min elution showing an absorbance peak at 253 nm and a shoulder at 275 nm, consistent with the profile of Q-modification (Costa et al., *J. Chromatography B*, 801, 237–247, 2004). (C) MALDI of the 21.586 min peak showing a m/z of 653.1 consistent with 5' phosphate of galatose-Q (galQ, $tRNA^{Tyr}$, top), and mannose-Q (manQ, $tRNA^{Asp}$, bottom).

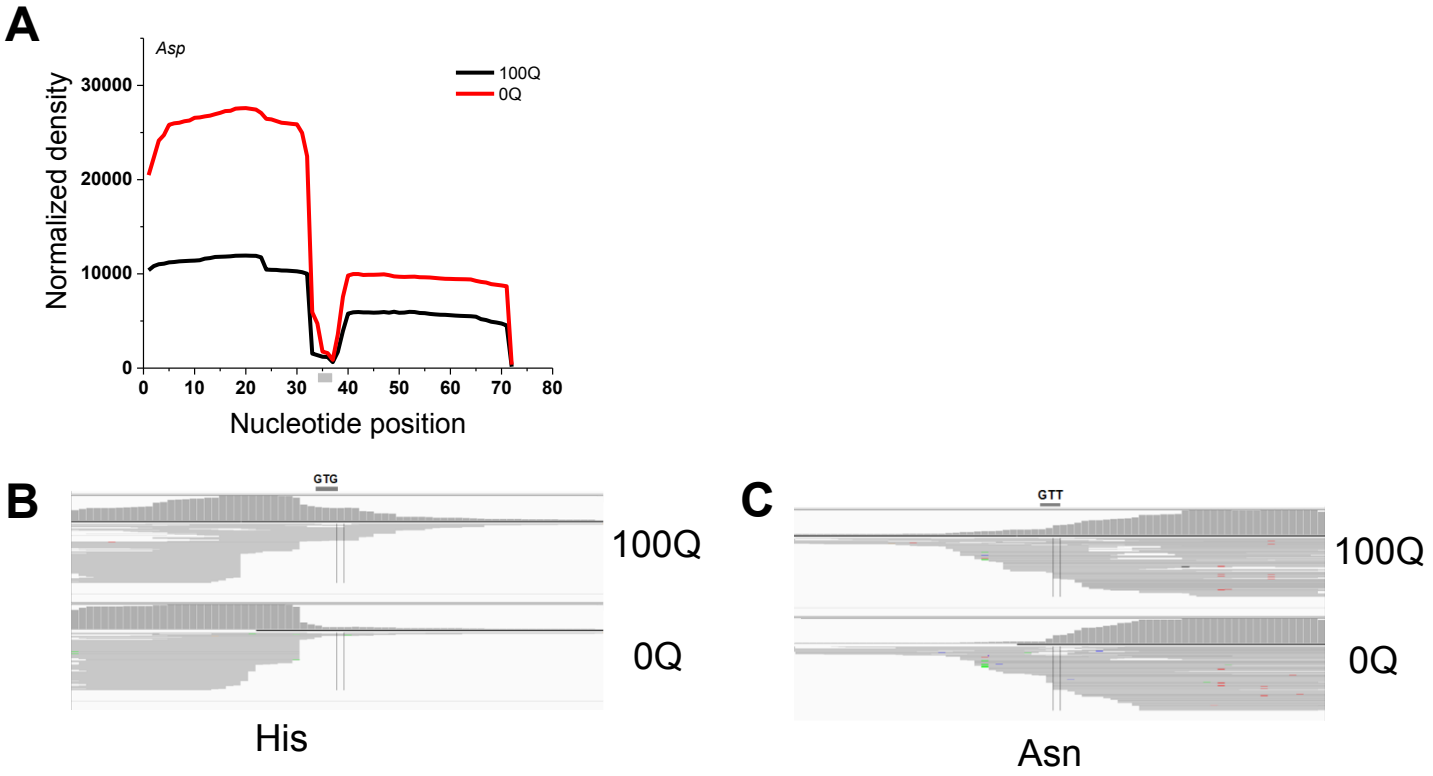
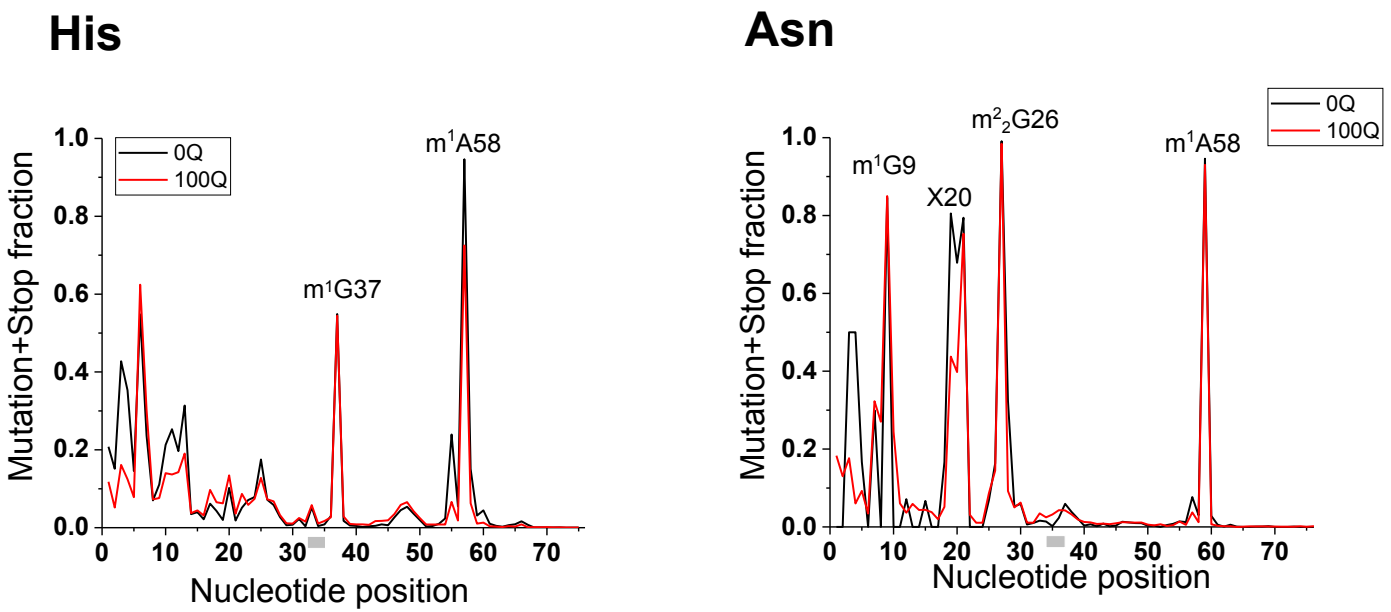


Fig. S3. Additional tRNA fragment sequencing results. (A) Density plot of $tRNA^{Asp}$ after normalization to all tRNA fragments in the individual samples. $tRNA^{Tyr}$ mapping is less reliable because of the highly abundant modifications in this tRNA. (B, C) IGV plots of $tRNA^{His}$ (B) and $tRNA^{Asn}$ (C) fragments from 0Q and 100Q cells showing common and distinct tRNA fragment patterns.



His (HeLa): D=dihydro U; P=pseudoU; K=m^1G; ?=m5C; "=m^1A.
GGCCGUGAUCGUAPAGDGGDDAGUACUCUGCGPUG(Q)UGG(K)CCGCAGCAA??UCGGUPCG"AUCCGAGUCACGGCACCA

Asn (Liver, placenta): D=dihydro U; P=pseudoU; 7=m7G; 6 = t6A; K=m^1G; ?=m5C; "=m^1A, R=$m^2{}_2G$, X=acp^3U.
GUCUCUGUKLCGCAADCGGDXAGCGCRPPCGGCUG(Q)UU6ACCGAAAG7DUGGUGGTPCG"GCCCACCCAGGGACGCCA

Fig. S4. Q-modification does not affect other modifications detectable by DM-tRNA-seq in $tRNA^{His}$ and $tRNA^{Asn}$. Shown are the sum of mutation and stop fraction at each position along $tRNA^{His}$ (left) and $tRNA^{Asn}$ (right). All modifications that interfere with Watson-Crick base pairing can be detected by this method (Clark et al., *RNA* 22, 1771-1784, 2016). The mutation+stop fraction is a proxy for modification fraction which can be compared quantitatively between two RNA samples. The known human $tRNA^{His}$ and $tRNA^{Asn}$ modifications (from Modomics) are also shown: anticodons are underlined, and the detectable modifications by sequencing are in red.

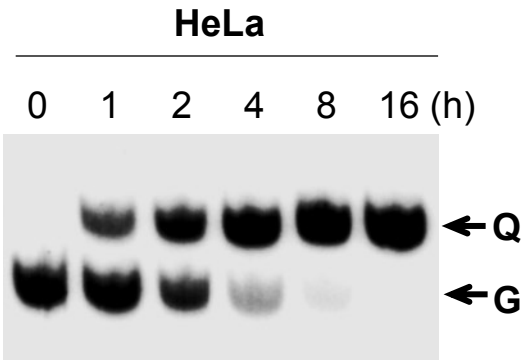


Fig. S5. Q-modification kinetics of HeLa cells.
Time course of Q modification for $tRNA^{Asn}$ starting with HeLa 0Q cells. Q and G indicate tRNA with Q34 and G34, respectively.

Table S1: Percent cleavage product of Q-modified cognate tRNAs by angiogenin cleavage *in vitro* using Northern blot.

$tRNA^{His}$	-CCA	3'halfmer	5'halfmer
0Q, -	5.9	n.d.	n.d.
100Q, -	11.9	n.d.	n.d.
0Q, +Ang	40.6	4.9	2.5
100Q, +Ang	62.2	3.3	1.0
$tRNA^{Asn}$			
0Q, -	3.9	n.d.	n.d.
100Q, -	4.8	n.d.	n.d.
0Q, +Ang	35.7	5.9	3.4
100Q, +Ang	54.5	1.0	0.2

n.d.: not detected.