Codon usage regulates human KRAS expression at both transcriptional and translational levels

Received for publication, July 16, 2018, and in revised form, September 17, 2018 Published, Papers in Press, October 1, 2018, DOI 10.1074/jbc.RA118.004908

© Jingjing Fu, Yi Liu

From the 1Department of Physiology, University of Texas Southwestern Medical Center, Dallas, Texas 75235, the 5State Key Laboratory for Conservation and Utilization of Bio-Resources and Center for Life Science, School of Life Sciences, Yunnan University, Kunming 650500, China, the 6Department of Pharmacology and Cancer Biology, School of Medicine, Duke University, Durham, North Carolina 27708

This work was supported by National Institutes of Health Grant 1R35GM118118, Cancer Prevention and Research Institute of Texas Grant RP160268, Welch Foundation Grant I-1560 (to Y. L.), and National Institutes of Health Grants R01CA94184 and P01CA203657 (to C. C.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains Figs. S1–S6.

The RAS family of small GTPases, comprised of KRAS, HRAS, and NRAS, is important for normal development and in cancer (1–4). With regards to the latter, these genes are collectively mutated in one-third of all human cancers (5). The encoded proteins share ∼85% homology, with the primary differences lying in the last ∼23 amino acids (6). Despite this high homology, multiple lines of evidence suggest that KRAS is expressed at low levels, and moreover, that this low expression is biologically critical to the function of the gene. Namely, KRAS mRNA levels are typically the lowest of the RAS isoforms in human tissues (7) (and see below). Furthermore, increasing the expression of the endogenous murine Kras gene results in hyperproliferation of their hematopoietic stem cells and renders the mice more resistant to a carcinogen that induces Kras-mutant lung tumors (8, 9). The importance of the low expression of KRAS cannot be overstated, as this gene is essential (10), unlike NRAS and HRAS, and is mutated in one quarter of all human cancers, far more than the other two RAS genes (4). In addition, overexpression or amplification of KRAS, but not mutations in the coding sequence, is also associated with certain types of cancer (11, 12). As such, elucidating the mechanism by which KRAS expression is kept low is critical to our understanding of normal and cancer biology.

Most amino acids are encoded by two to six synonymous genetic codons. Synonymous codons are used with different frequencies in all organisms, and every organism has its own preferred codon usage bias (13–16). Codon usage bias has been shown to positively correlate with tRNA abundance, thus optimal codons were thought to be translated more efficiently and more accurately (16–20). Consistent with this, strong codon usage biases have been shown to be important for the expression of highly expressed genes in different organisms, and codon optimization has been widely used to enhance heterologous protein expression (21–25). Therefore, codon usage can be an important determinant in gene expression. In addition, codon usage has been shown to influence the rate of translation elongation and protein structure by affecting the co-translational folding process in Escherichia coli, fungi, and insects (26–35). In addition to its role in regulating protein translation, codon usage also has a major role in determining the level of gene expression through transcriptional and post-transcriptional processes (24, 36–38). As such, gene codon usage has been proposed to be a code within the genetic code that can determine both gene expression levels and protein structures and therefore activity (24, 26).

Interestingly, whereas the RAS proteins share high amino acid identity, their codon usage varies widely. Human codon usage has biases for C/G at the wobble positions. HRAS is enriched in optimal codons, KRAS is enriched in rare codons and has a low codon bias score, whereas NRAS has a codon usage profile between these two (39). With regards to KRAS, we previously found that changing rare codons to common in the ectopic and/or endogenous human and/or murine KRAS increased KRAS mRNA, translation, and protein levels (39).
Regulation of KRAS expression by codon usage

Moreover, such changes had in vivo phenotype, namely altering the proliferation of hematopoietic stem cells or affecting various stages of tumorigenesis (9, 39, 40). However, the contribution of codon bias to the various steps in KRAS protein production had not been systematically examined. We now report that changing rare codons to common in KRAS increased translation efficiency and mRNA levels. Regulation of mRNA levels is a major mechanism affecting KRAS levels, but the effect was not a consequence of mRNA stability, but instead transcriptional. Moreover, codon usage also had an impact on the structure of KRAS. Thus, the rare codon bias of KRAS affects multiple aspects of gene expression process, which sheds important insights into the codon usage-mediated gene regulatory mechanisms for mammalian genes.

Results
Codon usage strongly affects expression of KRAS

Calculation of the average codon adaptation index (CAI)\(^2\) of human genes showed that the median CAI of human genes is 0.83. The average CAI of human HRAS is 0.87, whereas KRAS, which uses many rare codons, has an average CAI of 0.69 (39) (Fig. S1A). For example, GTG and ATC are the most preferred codons for valine and isoleucine, respectively, in the human genome. Although these optimal codons are overwhelmingly used in HRAS, they are rarely used in KRAS (Fig. S1B). Similar to our previously reported (39) data, transient transfection of an N terminally FLAG-tagged WT HRAS or KRAS cDNA (referred to as Hras and Kras constructs, respectively) in human embryonic kidney HEK-293T cells resulted in highly divergent protein expression (Fig. 1A). However, as noted above, codon bias can affect the entire process of protein production, and hence we decided to dissect the contribution of each to the ultimate end product of a functional protein. To this end, we first needed to benchmark the effect of the rare codons on KRAS expression.

We thus chose three previously created versions of human KRAS cDNA for this analysis (39). The WT KRAS was used as the control for native codon usage. In opKRAS, the rarest valine codon (GTA) was changed to the most common (GTG), but isoleucine codons encoding the N-terminal, central, or C-terminal regions were optimized (Fig. 1B and Fig. S2). These three versions of opKRAS expressed KRAS protein at levels intermediate between those of the Kras and opKrAs constructs (Fig. 1D). We also mutated the opKras construct so that only nine rare isoleucine or nine rare valine codons (opKras-I and opKras-V) were optimized. Both opKras-I and opKras-V resulted in KRAS protein levels that were higher than those produced from the WT Kras but lower than those from opKras (Fig. 1E). Thus, the three versions of KRAS exhibited the expected effect on protein production and different synonymous codons have independent and accumulative effects on KRAS expression.

Rare codons suppress KRAS mRNA translation

Codon usage has been shown to regulate translation elongation rate and co-translational protein folding (26, 29, 41) and has been proposed to influence translation efficiency and accuracy (16–20, 42). In agreement, we previously reported that changing rare codons to common increased KRAS mRNA in the polysomal fractions (39). To determine the effect of codon usage on KRAS translation, we synthesized different versions of KRAS mRNA by in vitro transcription; mRNAs had 5’ caps and poly(A) tails. Equal amounts of KRAS mRNA and the control luciferase mRNA were co-transfected into 293T cells and the amount of KRAS protein produced was determined. The amount of protein produced from opKRAS and KRAS\(^*\) mRNAs was about 0.5- and about 4-fold higher, respectively, than that produced from the WT KRAS mRNA (Fig. 2A). In contrast, the levels of luciferase protein were comparable (Fig. S5A).

Comparison of the protein decay rates after the addition of the protein synthesis inhibitor cycloheximide revealed that codon optimization in opKRAS and KRAS\(^*\) did not affect KRAS protein stability (Fig. S4). We note that the differences in protein levels using KRAS mRNA were much less than those observed in Fig. 1C, suggesting that additional mechanism(s) mediate the codon usage effect on protein production.

To further confirm the effect of codon usage on translation, we performed in vitro translation assays using 293T cell lysates. Similar fold-differences in KRAS protein production were observed for the opKRAS and KRAS\(^*\) mRNAs (Fig. 2B) as were observed in the above cell-based assay. Interestingly, when the same mRNAs were translated in an extract made from budding yeast, an organism with A/T-biased codons, the trend in expression pattern was opposite. The highest level of KRAS was synthesized from the WT KRAS mRNA (Fig. 2C and Fig. S5B). Together, these results suggest that rare codons in KRAS suppress KRAS translation in human cells. Furthermore, we generated stably transfected HEK-293T cell lines with Kras and with Kras\(^*\) expression constructs and performed polysome profiling (Fig. S5C). Northern blot analysis of the polysomal fractions showed that the WT Kras mRNA was peaked in the

---

2 The abbreviations used are: CAI, codon adaptation index; CTD, carboxy-terminal domain; eGFP, enhanced green fluorescent protein; CHX, cycloheximide.

17930 J. Biol. Chem. (2018) 293(46) 17929–17940
monosome fraction (fraction number 8), whereas Kras* mRNA was enriched in the polysome fractions (fractions 15–19) (Fig. S5C). Again, these data indicate that optimal codons of KRAS promote efficient mRNA translation in cells. However, we estimate a ~4-fold increase in translation (Fig. 2, A and B), which when benchmarked against the very high amount of protein produced by KRAS* suggest that translation is only one aspect accounting for the effect of rare codons on KRAS protein levels.

**KRAS codon optimization increases mRNA level but does not affect mRNA stability**

As noted above, multiple experiments support that rare codons impact KRAS protein production beyond translation. We had previously reported that ectopic KRAS* only generated an modest increase of mRNA compared with WT KRAS, as assessed by quantitative RT-PCR (39). To more accurately measure the effect of codon usage of KRAS mRNA levels we utilized Northern hybridization analysis using a probe that anneals to the common 5’ UTR of all three ectopic KRAS mRNAs. In cells that were transfected with different versions of KRAS ORF inserted in the expression construct. All constructs share the same promoter, 5’ and 3’ UTR. Lines marked the codon positions that were mutated.

![Figure 1. Codon usage determines KRAS expression levels. A, Western blotting results showing the levels of HRAS and KRAS in HEK-293 cells transiently transfected with cDNA expression construct. The polyvinylidene difluoride membrane used for Western blot analysis was stained with Amido Black to compare protein loading in different lanes. A prominent protein band stained by Amido Black (indicated as membrane) was shown to reflect protein loading. B, schematic diagram of different versions of KRAS ORF inserted in the expression construct. All constructs share the same promoter, 5’ and 3’ UTR. Lines marked the codon positions that were mutated. C–E, Western blotting results showing the protein level of KRAS in HEK-293 cells transfected with the indicated expression construct.](image-url)
Regulation of KRAS expression by codon usage

43–46). To determine whether the effect of codon usage on KRAS mRNA levels is due to its effects on mRNA stability, we compared mRNA decay rates of WT and codon-optimized mRNAs after the addition of α-amanitin, a commonly used transcription inhibitor. Northern blotting quantifications revealed that there were no significant differences in mRNA stability (Fig. 3B). A similar result was also obtained when another transcription inhibitor, actinomycin D, was used to determine mRNA decay rates (Fig. S6). These results indicate that in addition to its effect on translation efficiency, KRAS codon usage also has a major role in mRNA levels without overtly affecting mRNA stability. Consistent with this conclusion, it was previously shown that mammalian genes with high GC contents, which is associated with more preferred codons, have higher expression levels than those with lower GC content without affecting mRNA degradation rates (37, 47, 48).

KRAS codon usage regulates transcription and chromatin modifications

The increase in KRAS transcript levels by codon optimization prompted us to examine KRAS transcription. Using human 293T cell lines stably transfected with a vector encoding WT KRAS, opKRAS, or KRAS*, we performed Br-UTP-coupled nuclear run-on assays to examine transcriptional by RNA polymerase II (Pol II) at the locus of interest. Because this assay quantifies the frequency of transcription initiation, the levels of newly synthesized transcripts should not be influenced by RNA stability. Codon optimization resulted in significant increases of KRAS transcription with about 10-fold higher levels of transcript from Kras* than WT Kras (Fig. 4A). Quantification of KRAS transgene copy numbers in these stably transfected cell lines indicated that they have similar transgene copy numbers.

After transcriptional initiation, the Pol II carboxyl-terminal domain (CTD) is phosphorylated at serines 2 and 5 (49, 50). Ser-5 phosphorylation of the CTD tail occurs soon after initiation, whereas Ser-2 phosphorylation of the CTD of Pol II takes place during the transcriptional elongation process. To confirm the effect of codon usage on KRAS transcription, we compared the enrichment of phosphorylated Pol II CTD on the WT and codon-optimized KRAS by chromatin immunoprecipitation (ChIP) assays. Codon optimization resulted in a significant enrichment of both Ser-2 and Ser-5 phosphorylated Pol II at the
KRAS transgene loci (Fig. 4B). These results further indicate that codon usage impacts KRAS transcription.

To determine the mechanism by which codon usage affects KRAS transcription, we first performed histone H3 ChIP. The occupancies of histone H3 at the KRAS transgene loci showed no significant differences among three stable cell lines (Fig. 4C), suggesting that codon usage does not influence nucleosome density. We then performed ChIP assays for several histone modification marks associated with transcriptionally active chromatin. Both H3K4 trimethylation and H3K9 acetylation enrichments at the opKRAS and KRAS* were significantly increased compared with the WT KRAS transgene locus (Fig. 4D), consistent with mRNA level differences. p300 is the major histone acetyltransferase that mediates H3K9 acetylation. ChIP assays showed that the enrichment of p300 at the transgene loci was significantly higher for opKRAS cells than for the WT KRAS cells and was further increased for the KRAS* cells (Fig. 4E). These results indicate that the KRAS codon usage impacts...
transcription by affecting histone modifications and chromatin structure. Optimal codons may result in transcriptionally permissive chromatin structures to promote recruitment of transcription co-activators, such as p300.

To determine whether the effect of codon usage on transcription is a general phenomenon or is KRAS-specific, we examined the effect of codon usage on CFL2 expression. CFL2 encodes an intracellular protein that is a major component of intranuclear and cytoplasmic actin rods. Mutation of CFL2 results in human nemaline myopathy. Aside from its importance in human disease, we previously reported that CFL2 is enriched in rare codons, and that changing rare codons to common increases the amount of ectopic CFL2 protein (39). We confirmed that CFL2 protein expression is indeed greatly enhanced after codon optimization (Fig. 4F). Similar to KRAS, codon optimization also led to a 6-fold increase of CFL2 mRNA levels (Fig. 4G). ChIP assays were performed to examine the enrichment of Pol II Ser-2 and Ser-5 phosphorylation and H3K9 acetylation at the transgene loci in cells that stably expressed either the WT CFL2 or codon-optimized CFL2 (Fig. 4H). As expected, codon optimization resulted in a significant increase of enrichment of all three markers. Collectively, we conclude that codon usage also affects KRAS transcription and mRNA levels, an effect that may be applicable to other human genes enriched in rare codons.

**The differential effects of codon usage on KRAS expression in different cell lines**

We had previously demonstrated that ectopic expression of KRAS* generated more protein than KRAS in a variety of cell types (9, 39, 40). However, the contribution of changing rare codons to common on KRAS mRNA levels in different cells,
especially in light of the above results, had not been undertaken. To determine whether the effect of codon usage on \( \text{KRAS} \) mRNA levels is cell line-specific, we transfected the WT \( \text{KRAS} \), opKRAS, and \( \text{KRAS}^* \) expression constructs into two human hepatocellular carcinoma cell lines, Huh7 and HepG2, and two human breast cancer cell lines MDA-MB-231 and MCF-7. Codon optimization resulted in increases of both \( \text{KRAS} \) protein and mRNA in each of these cell lines (Fig. 5, A–D, and Fig. S7).

As in 293T cells, the fold-changes of mRNA levels due to codon optimization in these cell lines were smaller than those of protein levels, suggesting that the effect of codon usage on translation efficiency is shared among these cell lines. However, it is clear that different cell lines responded differently to codon optimization. In HepG2 cells, the effects of codon usage on \( \text{KRAS} \) protein and RNA were much smaller than those in the other cell lines. Less than 10-fold more \( \text{KRAS} \) protein and less than 50% more mRNA were produced from \( \text{KRAS}^* \) than from WT \( \text{KRAS} \). In contrast, in Huh7 cells the differences were larger than those seen in HEK-293T cells. In addition, the changes of \( \text{KRAS} \) transcript levels showed a strong positive correlation with the changes in protein levels in different cell lines (coefficient \( r = 0.93 \), Fig. 5E), indicating a major role for \( \text{KRAS} \) mRNA in determining \( \text{KRAS} \) protein levels. This suggests the intriguing possibility that the effect on codon bias at individual steps of protein production may be differentially regulated in different tissues. It should be noted that tRNA content can also be different in these cell lines which may affect translation efficiency (51).

**Codon optimization alters \( \text{KRAS} \) protein structure**

We and others have previously shown that codon usage affects the translation elongation rate in fungi and flies (26–28), which in turn can influence protein structure during the cotranslational folding process. Codon usage has been shown to regulate protein folding in vitro and in \( \text{E. coli} \), fungi, and \( \text{Drosophila} \) cells (26, 27, 29–35). To determine whether codon usage influences protein folding in mammals, we compared \( \text{KRAS} \) protein structures by performing a limited trypsin digestion assay, which can indicate protein structural differences. Cell extracts of 293T cells transfected with the WT \( \text{KRAS} \) or \( \text{KRAS}^* \) expression constructs were used for trypsin digestion assays. In the presence of the same concentration of trypsin, \( \text{KRAS} \) protein in the WT \( \text{KRAS} \) cell extract was much more resistant to trypsin digestion than that expressed from \( \text{KRAS}^* \) (Fig. 6A).

To confirm our conclusion, we carried out thermal shift assays using extracts of 293T cells (52, 53). This assay quantifies changes in thermal denaturation and aggregation temperature of a protein as a result of treatment by increasing temperatures, which results in the disappearance of the protein from the

---

**Figure 5. Differential \( \text{KRAS} \) codon usage responses in different cell lines.**

A–D, left panels: Western and Northern blotting results showing the levels of \( \text{KRAS} \) protein and mRNA levels in HepG2, Huh7, M231, and MCF-7 cell lines transiently transfected with the indicated \( \text{Kras} \) expression construct. Right panel, densitometric analyses of the Western and Northern blotting results for each cell line. Error bars denote mean ± S.D. E, scatter analysis showing the correlation between mRNA fold-changes and log\(_0\) of protein fold-changes. Pearson’s \( r = 0.93 \).
Regulation of KRAS expression by codon usage

supernatant. Changes in denaturation and aggregation temperature are indicative of structural changes of a protein. Increasing temperatures from 4 to 52.6 °C resulted in a gradual slow disappearance of KRAS from the WT KRAS extract. In contrast, most of the KRAS from the KRAS* extract was precipitated and disappeared from the supernatant above 40 °C (Fig. 6B). Together, these results suggested that codon optimization of KRAS alters structural properties of KRAS proteins.

Discussion

Unlike HRAS and NRAS, KRAS is an essential gene (10). However, the expression level of KRAS is much lower than that of HRAS in all tissues examined (Fig. 3C). As stated above, numerous experiments support the conclusion that the low level of KRAS is important for how the gene functions in normal and cancer biology of mammals, including in whole animal settings. Thus, the expression of KRAS is likely normally suppressed to prevent cancer in normal cells. As such, understanding how KRAS expression is maintained at a low level is critical. One feature of this gene that contributes to the poor expression of KRAS is due to its poor codon usage. Consistent with our previous observations, we show here that codon usage has a remarkable effect on KRAS expression from cDNA constructs. Codon optimization of KRAS resulted in the up-regulation of KRAS by about 100-fold (Fig. 1). In addition, the effect of codon usage was accumulative. The number of codons optimized was correlated with levels of KRAS, and effects were not restricted to a specific region of KRAS ORF. These results suggest that the different codon usage profiles of KRAS and HRAS are the primary reason for their different protein levels. In agreement with our previous observations, this increase was attributed to increased translation and mRNA levels. At the translational level, optimal codons promote efficient translation of KRAS mRNA. This conclusion is supported by analyses of translation of KRAS mRNA constructs in cells and in vitro (Fig. 2, A and B) and by polysome profiling results that showed that codon optimization of KRAS led to the enrichment of the KRAS mRNA in the highly translated polysome fraction. We have previously shown that optimal codons are known to increase the rate of translation elongation, and rare codons can result in ribosome stalling in fungi and fly cells (26–28). Our data suggest that codon usage has a similar effect in human cells.

Codon usage also determines KRAS mRNA levels. However, unlike in yeast and some other organisms (27, 38, 44–46), codon usage did not have a significant influence on KRAS mRNA stability in our experiments in human cells (Fig. 3B and Fig. S6). Instead, we found that optimal codon usage promotes KRAS transcription. This conclusion is supported by nuclear run-on and Pol II ChIP assay results (Fig. 4, A and B). In addition, we showed that codon optimization resulted in increases of H3K4me3 and H3K9ac, histone modifications that are associated with active chromatin (Fig. 4D). Furthermore, we found that codon optimization led to enrichment of histone acetyltransferase p300 at the KRAS locus. Similar effects of codon optimization were also observed for the CFL2 gene (Fig. 4, F–H), indicating that the transcriptional effect of codon usage may be a general phenomenon for human genes. Together, these results suggest that optimal codon usage affects transcription by recruiting co-transcriptional activators such as p300, which lead to chromatin modifications that alter chromatin structure and activate transcription. Preferred codons may enhance exonic transcription factor binding and thus affecting chromatin structure and transcription (54). Some of the experiments in this study were performed transiently in plasmid transfection. It should be noted that it was previously shown that transiently transfected plasmid DNA does form chromatin with histone proteins in cells (55, 56).

Consistent with our conclusion here, mammalian genes with high GC content, which is associated with more common codons, had higher expression levels than those with lower GC content without an effect on mRNA degradation rates (47, 48). In addition, codon usage was shown to contribute to the balanced expression of Toll-like receptors in mammals through effects on transcription (37). Codon usage was previously shown to have a major role at the translational level in *Neurospora* through regulation of chromatin structures (24). Therefore, a role of codon usage on transcription appears to be conserved from fungi to human. How codon usage affects chromatin structures is not known. Codon information within the ORF may be read by the transcription machinery in the form of DNA elements that favor or inhibit the recruitment of regulatory proteins that can regulate chromatin structure, resulting in suppression or activation of transcription. It should be noted that our experiment here was performed with ectopic expression from cDNA transgenes, which may have different effects from the endogenous locus. However, we have previously shown that KRAS codon optimization at the endogenous locus resulted in elevated KRAS expression (39).

Although a positive role for codon optimization on KRAS expression was observed in different cell lines, the degree of the codon usage effect differed (Fig. 5). Such differential codon usage effects may be caused by different tRNA expression profiles in different cell lines (51), which are known to influence translation. In addition, the effects may also be due to differential expression levels of the chromatin regulatory factors that mediate the transcriptional effect of codon usage.

Finally, our results show that KRAS codon usage may also affect KRAS protein structure. We and others have previously shown that codon usage regulates translation elongation in fungi and *Drosophila* (26–28). Changes in elongation rate change the time available for co-translational folding thus influencing protein structures. Consistent with this, codon usage has been shown to regulate protein folding in *in vitro* and in *E. coli*, fungi, and *Drosophila* cells (26, 27, 29–35). Our results suggest that this is also the case in human cells. Consistent with this conclusion, a single synonymous SNP that results in a rare codon in the human *MDR1* gene, which encodes a transporter, was found to result in altered drug and inhibitor interactions (57). Furthermore, codon usage has been implicated in co-translational protein folding of cystic fibrosis transmembrane conductance regulator (CFTR), a protein that regulates transmembrane conductance, which is mutated in cystic fibrosis patients (58–60). Taken together, our results demonstrate that codon usage influences gene expression and protein structure in human cells by multiple mechanisms. Because many human diseases are known to be associated with synonymous muta-
tions (11, 61, 62), our study suggests how these mutations can contribute to disease progression without altering amino acid sequences.

**Experimental procedures**

**Calculation of CAI**

CAI was calculated as previously described (63). Codon usage frequency table for *Homo sapiens* was obtained from [http://www.kazusa.or.jp/codon](http://www.kazusa.or.jp/codon) (65).

**Cell lines**

HEK-293T, HepG2, MDA-MB-231, and MCF7 cells were maintained at 37°C in 5% CO2 in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum (Sigma) and 100 units/ml of penicillin and streptomycin. HuH7 cells were maintained in McCoy’s 5A media (Gibco/Invitrogen) with identical supplements. For expression studies, cells were either transiently transfected with plasmids using PolyJet (SignaGen) according to the manufacturer’s instructions or selected for stable expression of constructs by puromycin or GVS North America. The membrane (GVS North America). The levels of eGFP were determined by Western blotting and fluorescence microscope analyses (Fig. S3).

**Plasmids**

pBabe-Kras, -opKras, and -Kras+ expression constructs were created previously (39). The other constructs used in this study were created based on these plasmids. The cDNA sequences of different codon-optimized KRAS constructs generated in this study are shown in Fig. S2.

**Protein and protein analysis**

Cells were lysed in RIPA (25 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM DTT, 0.5 mM PMSF, 10 µg/ml of aprotinin, 5 µg/ml of leupeptin, 1 µg/ml of pepstatin A, 25 mM NaF). Primary and secondary antibodies were used at the following dilutions: anti-FLAG, 1:2000 (Sigma); anti-myc, 1:5000 (Invitrogen); and anti-mouse HRP, 1:2000 (Bio-Rad). SDS-PAGE gels (12.5%) were used to resolve RAS proteins. For protein stability assays, cells were grown and transfected with the indicated plasmids for 2 days before the addition of CHX (final concentration 10 µg/ml) or α-amanitin (final concentration 50 µg/ml), and collected at the indicated time points.

**RNA analysis**

RNA was extracted with TRIzol (Ambion) in accordance with the manufacturer’s protocol. For Northern blotting analyses, equal amounts of total RNA (5 µg) were loaded onto agarose gels. After electrophoresis, the RNA was transferred onto the nitrocellulose membrane (GVS North America). The membrane was probed with an RNA probe specific for 5′ UTR of the KRAS mRNAs. The probe was labeled with [32P]UTP (PerkinElmer Life Technologies) during transcription by T7 RNA polymerase (Ambion) with the manufacturer’s protocol. The primer sequences used for the template amplification were Southern forward: 5′-CCTTATGTATCGTGGAGAGAAT-3′, and Southern reverse: 5′-TAAACTGACCTATAG-GGTCGTATCGTTTGTAGTC-3′.

For quantitative real-time PCR analysis, 500 ng of total RNA were reverse transcribed using a High-capacity cDNA Reverse Transcription Kit (ABI) in accordance with the manufacturer’s instructions and subjected to real-time PCR analysis. Primer sequences used for quantitation are as follows: ras forward: 5′-AGCCCTTGTGAACCCCTA-A-3′, ras reverse: 5′-GTGTC-TATCGTCTTTTGATGC-3′; gapdh forward: 5′-CATGGTCTCATGATGGTTGAACCA-3′, gapdh reverse: 5′-AGTGATGCGATGACTGTGATC-3′.

For the RNA stability assay, cells were grown and transfected with the indicated plasmids for 2 days before the addition of actinomycin D (final concentration 10 µg/ml) or α-amanitin (final concentration 50 µg/ml), and collected at the indicated time points.

**Nuclear run-on assay**

Cells were lysed in lysis solution (10 mM Tris-HCl, pH 7.4, 3 mM MgCl2, 10 mM NaCl, 150 mM sucrose, and 0.5% Nonidet P-40). Nuclei were isolated and suspended in 150 µl of storage buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM MgCl2, and 0.1 mM EDTA) and 150 µl of 2X transcription buffer (300 mM KCl, 10 mM Tris-HCl, pH 8, 5 mM MgCl2, 1 mM DTT, 500 µM ATP, 500 µM GTP, 500 µM Br-UTP, 2 µM CTP, and 200 units/ml of Superscript-in). After incubation at 37°C for 30 min, 6 µl of stop buffer and 60 units of RNase-free DNase I were added. RNAs were isolated using TRIzol. To isolate the newly synthesized RNA, Protein G beads were added, and incubated for 2 h at 37°C. Beads were washed and RNAs were extracted using TRIzol. Newly synthesized mRNA levels were measured by quantitative real-time PCR analysis. The primer set located in the 5′-UTR of KRAS that is shared among three transgene genes was used for quantitative RT-PCR: Kras forward: 5′-AGCCCTTGTGACCCCTA-A-3′, reverse: 5′-GTGTCATCATGATGGTTGAACCA-3′. The results were normalized to GAPDH mRNA expression levels in each sample and further normalized to mRNA levels at the endogenous Kras locus.

**ChIP assay**

Cells were fixed with 1% formaldehyde (Sigma) for 15 min at room temperature with shaking. Glycine (Sigma) was then added to a final concentration of 125 mM. The cross-linked cells were collected and prepared using lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS, Roche complete protease inhibitor (EDTA-free)) with sonication. Equal amounts of protein were used for each immunoprecipitation reaction. Antibodies against histone H3 (ab1791), the RNA Pol II C-terminal domain (phospho-S2; ab5095), the RNA Pol II C-terminal domain (phospho-S5; ab5131), and histone H3 (trimethyl-K4, ab8580) were purchased from Abcam. Antibody against histone H3 acetyl-Lys-9 (39917) was purchased from Active Motif. Antibody against p300 (sc-48343) was purchased from Santa Cruz Biotechnology. The ChIP reaction was carried out with 2 µl of antibody. Immunoprecipitated DNA was enriched using...
In vitro transcription

To prepare the templates for in vitro transcription, the plasmids were linearized by NheI followed by successive phenol-chloroform extraction and ethanol precipitation. The capped and poly(A)-tailed mRNA transcripts were synthesized using a HiScribe T7 quick high yield RNA synthesis kit (New England Biolabs) supplemented with 3’-o-Me-m7G(5’)-ppp(5’)G antisense cap structure analog (New England Biolabs) following the manufacturer’s instructions. The mRNA concentrations were measured using a Nanodrop (Thermo Scientific).

In vitro translation using mammalian HEK-293T and Saccharomyces cerevisiae cell-free lysates

To prepare HEK-293T cell-free lysate, HEK-293T cells were harvested by centrifugation at 1000 × g for 4 min and washed with PBS three times. Cell pellets were resuspended in 2 volumes of hypotonic buffer (10 mM HEPES-KOH, pH 7.6, 10 mM potassium acetate, 0.5 mM magnesium acetate, 5 mM DTT), and incubated on ice for 40 min to 1 h. Cells were then homogenized by 20–30 strokes in a Dounce homogenizer on ice, and the final concentration of potassium acetate was adjusted to 50 mM. The cell extract was centrifuged at 16,000 × g for 10 min at 4 °C. The supernatant was aliquoted, snap frozen in liquid nitrogen, and stored at −80 °C before use. To perform translation assay, 3 μl of reaction mixture (20 mM HEPES-KOH, pH 7.6, 0.5 mM spermidine, 8 mM creatine phosphate, 0.2 mM GTP, 1 mM ATP, 20 μM complete amino acids (Promega), 100 mM potassium acetate, 1 mM magnesium acetate, 0.13 units/μl of creatine phosphate kinase, 0.2 units/μl of SUPERaseIn RNase Inhibitor (Invitrogen)), 1 μl of the mRNA template (180 ng), and 8 μl of cell-free translation extract were used in each reaction. The reactions were incubated in a 30 °C water bath for 30 min and stopped by adding SDS sample buffer, followed immediately by heating at 90 °C. The samples were subsequently analyzed by Western blotting.

To prepare S. cerevisiae cell-free lysate, cells were harvested by centrifugation at 4 °C for 5 min at 3,000 rpm, and resuspended in 1.5 ml of buffer A (30 mM HEPES-KOH, pH 7.6, 100 mM potassium acetate, 3 mM magnesium acetate, 2 mM DTT) with 8.5% mannitol and 0.5 mM PMSF/g of cell weight (64). Lysate were centrifuged at 4 °C for 6 min at 18,000 rpm and supernatant was collected. Small molecular weight molecules are removed from the extract using Zeba Desalt Spin Columns (Pierce). Aliquots (200 μl) are pipetted into 1.6-ml Eppendorf tubes, frozen with liquid nitrogen, and stored at −80 °C. To perform translation assay, 7 μl of translation reaction mixture (5 μl of cell lysate with 1 μl of 10× energy mixture, 0.06 μl of 10 units of creatine phosphate kinase, 0.5 μl of 2 mM KOAc, 0.12 μl of 0.1 M Mg(OAc)2, 0.1 μl of 1 mM amino acids mixture, and 0.1 μl of SUPERaseIn RNase inhibitor (Life Technologies), and 0.12 μl of RNase-free water), and 3 μl of the mRNA template (60 ng) was used in each reaction. The reactions were incubated in a 26 °C water bath for 15 min and stopped by adding SDS sample buffer, followed immediately by heating at 90 °C. The samples were subsequently analyzed by Western blotting.

Thermal shift assay

Protein extract was diluted to a total protein concentration of 2.5 μg/μl. A 100-μl aliquot of extract was treated with trypsin at room temperature with gentle shaking. A 20-μl sample was taken from the reaction at each time point (0, 5, 15, and 30 min) after addition of trypsin. Each 20-μl sample was mixed with protein loading buffer, and proteins were resolved on an SDS-PAGE gel (12.5%). Western blotting was performed to examine KRAS protein levels at each time point as previously described (29). Experiments were performed side by side and protein samples were transferred to the same membrane for Western blot analysis.

Acknowledgments—We thank Dr. Chengcheng Zhang and co-workers for technical assistance, Drs. Hao Zhu and Weibo Luo for generously providing cell lines used in this study, and Sigi Li for comments to the manuscript.
Regulation of KRAS expression by codon usage

References

1. Cox, A. D., and Der, C. J. (2010) Ras history: the saga continues. Small GTPases 1, 2–27 CrossRef Medline

2. Colicelli, J. (2004) Human RAS superfamily proteins and related GTPases. Sci. STKE 2004, RE13 Medline

3. Pylayeva-Gupta, Y., Graboeck, A., and Bar-Sagi, D. (2011) RAS oncogenes: weaving a tumorigenic web. Nat. Rev. Cancer 11, 761–774 CrossRef Medline

4. Prior, J. A., Lewis, P. D., and Mattos, C. (2012) A comprehensive survey of Ras mutations in cancer. Cancer Res. 72, 2457–2467 CrossRef Medline

5. Cox, A. D., Fesik, S. W., Kimmelman, A. C., Luo, J., and Der, C. J. (2014) Drugging the undruggable RAS: mission possible? Nat. Rev. Drug Discov. 13, 828–851 CrossRef Medline

6. Barbacid, M. (1987) ras genes. Annu. Rev. Biochem. 56, 779–827 CrossRef Medline

7. Fiorucci, G., and Hall, A. (1988) All three human ras genes are expressed in a wide range of tissues. Biochim. Biophys. Acta 950, 81–83 CrossRef Medline

8. Sasine, J. P., Himburg, H. A., Termini, C. M., Roos, M., Tran, E., Zhao, L., Li, M., Zhang, Y., Barros, S. d., Rao, D. S., Counter, C. M., and Chute, J. P. (2018) Wild-type Kras expands and exhausts hematopoietic stem cells. J. Clin. Invest. Insight 3, pii 98197 Medline

9. Perspective, N. L., Lampson, B. L., Prinz, J. A., Lacsina, J. R., Marzluff, W. F., Otani, H., Aiba, A., and Katsuki, M. (1997) K-ras is essential for the development of the mouse embryo. Oncogene 15, 1151–1159 CrossRef Medline

10. Koera, K., Nakamura, K., Nakao, K., Miyoshi, J., Toyoshima, K., Hatta, T., Otani, H., Aiba, A., and Katsuki, M. (1997) K-ras is essential for the development of the mouse embryo. Oncogene 15, 1151–1159 CrossRef Medline

11. Birkenland, E., Wink, E., Mjas, S., Hoivik, E. A., Trovik, J., Werner, H. M., Hoivik, E. A., Mjøs, S., Trovik, J., Werner, H. M., and Birkeland, E. (2004) Regulation of KRAS expression by codon usage and other coding DNA-based features for prediction of protein expression in Saccharomyces cerevisiae. Yeast 21, 1083–1093 CrossRef Medline

12. Duret, L., and Mouchiroud, D. (1999) Expression pattern and, surprisingly, gene length shape codon usage in Caenorhabditis, Drosophila, and Arabidopsis. Proc. Natl. Acad. Sci. U.S.A. 96, 4482–4487 CrossRef Medline

13. Carlini, D. B., and Stephan, W. (2003) In vivo introduction of unpreferred synonymous codons into the Drosophila Adh gene results in reduced levels of ADH protein. Genetics 163, 239–243 Medline

14. Zhou, Z., Dang, Y., Zhou, M., Li, L., Yu, C. H., Fu, J., Chen, S., and Liu, Y. (2016) Codon usage is an important determinant of gene expression levels largely through its effects on transcription. Proc. Natl. Acad. Sci. U.S.A. 113, E6117–E6125 CrossRef Medline

15. Gustafsson, C., Govindarajan, S., and Minshull, J. (2004) Codon bias and heterologous protein expression. Trends Biotechnol. 22, 346–353 CrossRef Medline

16. Yu, C. H., Dang, Y., Zhou, Z., Wu, C., Zhao, F., Sachs, M. S., and Liu, Y. (2015) Codon usage influences the local rate of translation elongation to regulate codon-translation protein folding. Mol. Cell 59, 744–754 CrossRef Medline

17. Zhang, F., Yu, C. H., and Liu, Y. (2017) Codon usage regulates protein structure and function by affecting translation elongation speed in Drosophila cells. Nucleic Acids Res. 45, 8484–8492 CrossRef Medline

18. Weinberg, D. E., Shah, P., Eichhorn, S. W., Hussmann, J. A., Plotkin, J. B., and Bartel, D. P. (2016) Improved ribosome-footprint and mRNA measurements provide insights into dynamics and regulation of yeast translation. Cell Rep. 14, 1787–1799 CrossRef Medline

19. Zhou, M., Guo, J., Cha, J., Chae, M., Chen, S., Barral, J. M., Sachs, M. S., and Liu, Y. (2013) Non-optimal codon usage affects expression, structure and function of clock protein FRQ. Nature 495, 111–115 CrossRef Medline

20. Zhou, M., Wang, T., Fu, J., Xiao, G., and Liu, Y. (2015) Nonoptimal codon usage influences protein structure in intrinsically disordered regions. Mol. Microbiol. 97, 974–987 CrossRef Medline

21. Komar, A. A., Lesnik, T., and Reiss, C. (1999) Synonymous codon substitutions affect ribosome traffic and protein folding during in vitro translation. FEBS Lett. 462, 387–391 CrossRef Medline

22. Buhr, F., Jha, S., Thommen, M., Mittelstaedt, J., Kutz, F., Schwabe, H., Rodnina, M. V., and Komar, A. A. (2016) Synonymous codons direct cotranslational folding toward different protein conformations. Mol. Cell 61, 341–351 CrossRef Medline

23. Sander, I. M., Chaney, J. L., and Clark, P. L. (2014) Expanding Anfinsen’s principle: contributions of synonymous codon selection to rational protein design. J. Am. Chem. Soc. 136, 858–861 CrossRef Medline

24. Spencer, P. S., Siller, E., Anderson, J. F., and Barral, J. M. (2012) Silent substitutions predictably alter translation elongation rates and protein folding efficiencies. J. Mol. Biol. 422, 328–335 CrossRef Medline

25. Fu, J., Murphy, K. A., Zhou, M., Li, Y. H., Lam, V. H., Tabuloc, C. A., Chiu, J. C., and Liu, Y. (2016) Codon usage affects the structure and function of the Drosophila circadian clock protein PERIOD. Genes Dev. 30, 1761–1775 CrossRef Medline

26. Zhou, Z., Dang, Y., Zhou, M., Yuan, H., and Liu, Y. (2018) Codon usage biases co-evolve with transcription termination machinery to suppress premature cleavage and polyadenylation. Elife 7, e33569 CrossRef Medline

27. Newman, Z. R., Young, J. M., Ingolia, N. T., and Barton, G. M. (2016) Differences in codon bias and GC content contribute to the balanced expression of TLR7 and TLR9. Proc. Natl. Acad. Sci. U.S.A. 113, E1362–1371 CrossRef Medline

28. Presnyak, V., Alhusaini, N., Chen, Y. H., Martin, S., Morris, N., Kline, N., Olson, S., Weinberg, D., Baker, K. E., Graveley, B. R., and Coller, J. (2015) Codon optimality is a major determinant of mRNA stability. Cell 160, 1111–1124 CrossRef Medline

29. Lampson, B. L., Pershin, N. L., Prinz, J. A., Lacsina, J. R., Marzluff, W. F., Nicchitta, C. V., MacAlpine, D. M., and Counter, C. M. (2013) Rare codons regulate KRAs oncogenesis. Curr. Biol. 23, 70–75 CrossRef Medline

30. Ali, M., Kaltenbrun, E., Anderson, G. R., Stephens, S. J., Arena, S., Bardelli, A., Counter, C. M., and Wood, K. C. (2017) Codon bias imposes a targetable limitation on KRAS-driven therapeutic resistance. Nat. Commun. 8, 15617 CrossRef Medline
Regulation of KRAS expression by codon usage

41. Pechmann, S., Charton, J. W., and Frydman, J. (2014) Local slowdown of translation by non-optimal codons promotes nascent-chain recognition by SRP in vivo. Nat. Struct. Mol. Biol. 21, 1100–1105 CrossRef Medline

42. Qian, W., Yang, J. R., Pearson, N. M., Maclean, C., and Zhang, J. (2012) Balanced codon usage optimizes eukaryotic translational efficiency. PLoS Genet. 8, e1002603 CrossRef Medline

43. Boel, G., Letso, R., Neely, H., Price, W. N., Wong, K. H., Su, M., Luff, J., Valecha, M., Everett, J. K., Acton, T. B., Xiao, R., Montelione, G. T., Alaberts, D. P., and Hunt, J. F. (2016) Codon influence on protein expression in E. coli correlates with mRNA levels. Nature 529, 358–363 CrossRef Medline

44. Mishima, Y., and Tomari, Y. (2016) Codon usage and 3’ UTR length determine maternal mRNA stability in zebrafish. Mol Cell 61, 874–885 CrossRef Medline

45. Bazzini, A. A., Del Viso, F., Moreno-Mateos, M. A., Johnstone, T. G., Vejnjar, C. E., Qin, Y., Yao, J., Khokha, M. K., and Giraldez, A. J. (2016) Codon identity regulates mRNA stability and translation efficiency during the maternal-to-zygotic transition. EMBO J. 35, 2087–2103 CrossRef Medline

46. Radhakrishnan, A., Chen, Y. H., Martin, S., Alhusaini, N., Green, R., and Coller, J. (2016) The DEAD-box protein Dhh1p couples mRNA decay and translation by monitoring codon optimality. Cell 167, 122–132 e129 CrossRef Medline

47. Kudla, G., Lipinski, L., Caffin, F., Helwak, A., and Zylicz, M. (2006) High guanine and cytosine content increases mRNA levels in mammalian cells. PLoS Biol. 4, e180 CrossRef Medline

48. Krinner, S., Heitzer, A. P., Diermeier, S. D., Obermeier, I., Längst, G., and Wagner, R. (2014) Cpg domains downstream of TSSs promote high levels of gene expression. Nucleic Acids Res. 42, 3551–3564 CrossRef Medline

49. Komaritsky, P., Cho, E. J., and Buratowski, S. (2000) Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription. Genes Dev. 14, 2452–2460 CrossRef Medline

50. Hsin, J. P., and Manley, J. L. (2012) The RNA polymerase II CTD coordinates transcription and RNA processing. Genes Dev. 26, 2119–2137 CrossRef Medline

51. Dittmar, K. A., Goodenour, J. M., and Pan, T. (2006) Tissue-specific differences in human transfer RNA expression. PLoS Genet. 2, e221 CrossRef Medline

52. Martinez Molina, D., Jafari, R., Ignatuschchenko, M., Seki, T., Larsson, E. A., Dan, C., Sreekumar, L., Cao, Y., and Nordlund, P. (2013) Monitoring drug target engagement in cells and tissues using the cellular thermal shift assay. Science 341, 84–87 CrossRef Medline

53. Jafari, R., Almqvist, H., Axelsson, H., Ignatuschchenko, M., Lundbäck, T., Nordlund, P., and Martinez Molina, D. (2014) The cellular thermal shift assay for evaluating drug target interactions in cells. Nat. Protoc. 9, 2100–2122 CrossRef Medline

54. Stergachis, A. B., Haugen, E., Shafer, A., Fu, W., Vernot, B., Reynolds, A., Raubitschek, A., Ziegler, S., LeProust, E. M., Akkey, J. M., and Stamatoyannopoulos, J. A. (2013) Exonic transcription factor binding directs codon choice and affects protein evolution. Science 342, 1367–1372 CrossRef Medline

55. Liu, R., Liu, H., Chen, X., Kirby, M., Brown, P. O., and Zhao, K. (2001) Regulation of CSF1 promoter by the SWI/SNF-like BA complex. Cell 106, 309–318 CrossRef Medline

56. Mallon, S., Wakim, B. T., and Roizman, B. (2012) Use of biotinylated plasmid DNA as a surrogate for HSV DNA to identify proteins that repress or activate viral gene expression. Proc. Natl. Acad. Sci. U.S.A. 109, E3549–3557 CrossRef Medline

57. Kimchi-Sarfaty, C., Oh, J. M., Kim, I. W., Sauna, Z. E., Calcagno, A. M., Ambudkar, S. V., and Gottesman, M. M. (2007) A “silent” polymorphism in the MDR1 gene changes substrate specificity. Science 315, 525–528 CrossRef Medline

58. Kim, S. J., Yoon, J. S., Shishido, H., Yang, Z., Rooney, L. A., Barral, J. M., and Skach, W. R. (2015) Translational tuning optimizes nascent protein folding in cells. Science 348, 444–448 CrossRef Medline

59. Kirchner, S., Cai, Z., Rauscher, R., Kastelic, N., Anding, M., Czech, A., Kleizen, B., Osteggaard, L. S., Braakman, I., Sheppard, D. N., and Ignatova, Z. (2017) Alteration of protein function by a silent polymorphism linked to tRNA abundance. PLoS Biol. 15, e2000779 CrossRef Medline

60. Lazarak, A., Fu, L., Bali, V., Bartoszewski, R., Rab, A., Havasi, V., Keiles, S., Kappes, J., Kumar, R., Lefkowitz, E., Sorscher, E. J., Matalon, S., Collawn, J. F., and Bebok, Z. (2013) The silent codon change I507-ATC→ATT contributes to the severity of the Δ/F508 CFTR channel dysfunction. FASEB J. 27, 4630–4645 CrossRef Medline

61. Sauna, Z. E., and Kimchi-Sarfaty, C. (2011) Understanding the contribution of synonymous mutations to human disease. Nat. Rev. Genet. 12, 683–691 CrossRef Medline

62. McCarthy, C., Carrea, A., and Diambra, L. (2017) Bicodon bias can determine the role of synonymous SNPs in human diseases. BMC Genomics 18, 227 CrossRef Medline

63. Sharp, P. M., and Li, W. H. (1987) The codon Adaptation Index: a measure of directional synonymous codon usage bias, and its potential applications. Nucleic Acids Res. 15, 1281–1295 CrossRef Medline

64. Wu, C., Amrani, N., Jacobson, A., and Sachs, M. S. (2007) The use of fungal in vitro systems for studying translational regulation. Methods Enzymol. 429, 203–225 CrossRef Medline

65. Nakamura, Y., Gojobori, T., and Ikemura, T. (2000) Codon usage tabulated from the international DNA sequence databases: Status for the year 2000. Nucleic Acids Res. 28, 292 CrossRef Medline