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Regulation of mRNA export by the PI3 kinase/AKT signal transduction pathway

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ABSTRACT UAP56, ALY/REF, and NXF1 are mRNA export factors that sequentially bind at the 5′ end of a nuclear mRNA but are also reported to associate with the exon junction complex (EJC). To screen for signal transduction pathways regulating mRNA export complex assembly, we used fluorescence recovery after photobleaching to measure the binding of mRNA export and EJC core proteins in nuclear complexes. The fraction of UAP56, ALY/REF, and NXF1 tightly bound in complexes was reduced by drug inhibition of the phosphatidylinositol 3-kinase (PI3 kinase)/AKT pathway, as was the tightly bound fraction of the core EJC proteins elf4A3, MAGOH, and Y14. Inhibition of the mTOR mTORC1 pathway decreased the tight binding of MAGOH. Inhibition of the PI3 kinase/AKT pathway increased the export of poly(A) RNA and of a subset of candidate mRNAs. A similar effect of PI3 kinase/AKT inhibition was observed for mRNAs from both intron-containing and intronless histone genes. However, the nuclear export of mRNAs coding for proteins targeted to the endoplasmic reticulum or to mitochondria was not affected by the PI3 kinase/AKT pathway. These results show that the active PI3 kinase/AKT pathway can regulate mRNA export and promote the nuclear retention of some mRNAs.

INTRODUCTION

The export of RNA molecules from the nucleus to the cytoplasm is a critical step in cellular maintenance. The export mechanisms for different classes of RNA share a common approach; RNAs are packaged in messenger ribonucleoprotein (mRNP) complexes that bind export receptor subcomplexes that subsequently dock the complex at nuclear pores for translocation to the cytoplasm (Rodriguez et al., 2004; Stewart, 2007). The export receptor for most mRNAs is NXF1. In this export mechanism, the ATP-dependent RNA helicase UAP56 is deposited on mRNA during splicing. UAP56 recruits the adapter protein ALY/REF (Luo et al., 2001), which in turn binds the NXF1-p15 heterodimer that is required for most mRNA export in species from yeast to humans (Segref et al., 1997; Grütter et al., 1998; Herold et al., 2001).

In one model, this complex of UAP56, ALY/REF, and NXF1-p15 associates with a spliced mRNA at the capped 5′ end of the mRNA (Cheng et al., 2006). UAP56 and ALY/REF are members of the transcription/export (TREX) complex located at the 5′ end (Bensaude, 2011; Nechaev and Adelman, 2011). NXF1 recruited to the complex may subsequently bind at nuclear pores (Forler et al., 2004), and also to transportin-2 (Shamsher et al., 2002), for export of the mRNP complex from the nucleus.

A second complex, the exon junction complex (EJC), forms on the mRNA at sites 24 nucleotides upstream from exon–exon junctions (Singh et al., 2012) and could play a role in export to the cytoplasm. The crystal structure of the core EJC with elf4A3, MLN51, MAGOH, and Y14 bound to polyU has been solved (Andersen et al., 2006; Bono et al., 2006). UAP56, ALY/REF, and NXF1 are all reported to be members of an extended EJC (Kataoka et al., 2000; Le Hir et al., 2000). In vitro, at least three EJC proteins—UAP56, Y14, and MAGOH—bind to the export receptor NXF1-p15 heterodimer (Kataoka et al., 2001). Proteins interacting with the EJC also mediate the translational repression and nonsense-mediated decay of mRNAs containing premature stop codons (Singh et al., 2007; Isken et al., 2008).
The PI pathway is a central regulator of cell metabolism, gene expression, and development (Yuan and Cantley, 2008; Chalhoub and Baker, 2009; Skwirek and Boulainne, 2009). PI(4,5)P2 is the key pathway intermediate; it can be further phosphorylated by phosphatidylinositol 3-kinase (PI3 kinase), generating PI(3,4,5)P3, which is a potent activator of several downstream effectors, including AKT (Altomare and Testa, 2005). Surprisingly, PI(4,5)P2 is present in the nucleus at RNA-spooling speckled domains, which are enriched in mRNA-spooling and export factors (Boronenkov et al., 1998; Osborne et al., 2001; Stallings et al., 2005; Bunce et al., 2006). The nucleus of a typical cell has 20–40 RNA-spooling speckled domains around which transcription, RNA splicing, and RNA export from the nucleus are organized. A majority of, but not all, pre-mRNA transcripts are spliced at or near these domains (Smith et al., 1999), and some mRNAs move into the interior of these domains and bind before release to the cytoplasm (Shopland et al., 2002).

In this study we used fluorescence recovery after photobleaching (FRAP) to screen proteins present in mRNA export complexes for changes in binding after inhibition of the PI3K/AKT or mTOR signal transduction pathways. PI3 kinase and AKT did regulate the binding affinity of the essential mRNA export proteins UAP56 and NXF1 in complexes formed at RNA-spooling speckled domains and at sites in the nucleoplasm. The assembly of the core EJC proteins elF4A3, MAGOH, and Y14 into complexes at speckled domains was also altered by inhibition of PI3 kinase or AKT. MAGOH binding was strongly reduced by the inhibition of mTOR. The activated PI3K/AKT pathway caused increased retention of poly(A) RNA and specific mRNAs in the nucleus, consistent with this signal transduction pathway regulating the export of mRNA.

**RESULTS**

The binding of mRNA export proteins in live cells is regulated by PI3 kinase and AKT.

FRAP measures the relative kinetics and affinities of fluorescent protein-binding in complexes that are immobile over the time course of the experiment (Wagner et al., 2004; Lele et al., 2006; Lele and Ingber, 2006; Kota et al., 2008; Nickerson, 2009). To identify mRNA export factors whose assembly into export complexes is regulated by the PI signal transduction pathway, we screened enhanced green fluorescent protein (EGFP)-fusion proteins of known mRNA export factors for changes in binding after inhibition of the PI3K/AKT or mTOR signal transduction pathways. PI3 kinase and AKT did regulate the binding affinity of the essential mRNA export proteins UAP56 and NXF1 in complexes formed at RNA-spooling speckled domains and at sites in the nucleoplasm. The assembly of the core EJC proteins elF4A3, MAGOH, and Y14 into complexes at speckled domains was also altered by inhibition of PI3 kinase or AKT. MAGOH binding was strongly reduced by the inhibition of mTOR. The activated PI3K/AKT pathway caused increased retention of poly(A) RNA and specific mRNAs in the nucleus, consistent with this signal transduction pathway regulating the export of mRNA.

**FIGURE 1:** The binding of mRNA export complex proteins, as measured by FRAP, is regulated by PI3 kinase, AKT, and mTOR. HeLa cells were transfected with EGFP-fusion proteins for UAP56, ALY/REF, and NXF1. After 48 h, cells were treated with 20 μM of the PI3 kinase inhibitor LY29402 (green circles) for 2 h, 5 μM of AKT inhibitor VIII (blue triangles) for 3.5 h, and 100 nM of the mTOR inhibitor rapamycin (red squares) for 5 h. Multiple cells were examined by FRAP over a 2-h interval in all treatment groups. The times between drug addition and photobleaching are reported as a mean. Black squares, untreated cells. Normalized fluorescence recovery curves are presented for EGFP-UAP56, EGFP-TAP/NXF1, and EGFP-ALY/REF. Also shown is EGFP-UAP56 K95N, a point mutant that cannot bind ATP. Each colored arrow marks the t½ of recovery for the curve of the same color. Statistical analysis of the differences between averaged FRAP curves was by paired one-tailed t test: untreated EGFP-UAP56 vs. LY294002 (p = 0.0083), AKT (p < 0.0001), rapamycin (p = 0.2799); untreated EGFP-ALY/REF vs. LY294002 (p < 0.0001), AKT (p < 0.0001), rapamycin (p < 0.0001); untreated EGFP-NXF1 vs. LY294002 (p = 0.0002), AKT (p = 0.0004), rapamycin (p = 0.4102). Means are plotted with error bars for standard errors. n, number of cells in each treatment group.
which represents the tightly bound fraction that did not exchange during the time course of the experiment. After inhibition of PI3 kinase with LY294002 for 2–3 h, the recovery half-time increased to 21.2 s (Figure 1 and Table 1), and the tightly bound immobile fraction was markedly decreased from 27.7 to 17.1% (Figure 1 and Table 1). This immobile fraction represents the fraction of UAP56 bound in more stable complexes. The immobile fraction of EGFP-UAP56 was similarly reduced from 27.7 to 8.5% by inhibition of AKT with 5 μM Akt inhibitor VIII for 3–4 h (Vlahos et al., 1994; Barnett et al., 2005).

AKT is activated downstream of PI3 kinase by phosphorylation at threonine 308 (Alessi et al., 1996), but mTOR signaling through mTORC2 can also influence AKT activity (Nave et al., 1999) by phosphorylation of AKT at serine 473 (Sarbassov et al., 2005; Jacinto et al., 2006). Akt inhibitor VIII is an allosteric inhibitor of AKT 1 (IC_{50} = 58 nM), AKT2 (IC_{50} = 210 nM), and AKT3 (IC_{50} = 2119 nM; Lindsley et al., 2005) that inhibits the activating phosphorylations of both T308 and S473 (AKT1 numbering; Calleja et al., 2009). This gives this inhibitor greater specificity for AKT than other inhibitors that target the ATP-binding sites of AKT, sites that have conserved features with other ATP-binding proteins. The crystal structure of Akt inhibitor VIII inhibitor binding to AKT1 was solved (Wu et al., 2005). Akt inhibitor VIII reduces AKT kinase activity and has a dominant-negative effect on the export of a splicing reporter mRNA (Kota et al., 2008). A similar point mutant in UAP56 of Saccharomyces cerevisiae inhibited its function (Zhang and Green, 2001). In control experiments, EGFP-UAP56K95N was less concentrated at speckled domains and, after a photobleach, recovered with t_{1/2} = 2.6 s and a very small immobile fraction (5.8%), both consistent with low-affinity binding. For this mutant, there was no significant change in the photobleach recovery kinetics or in the immobile fraction caused by drug treatments (Figure 1 and Table 1).

We verified the effectiveness of the drug treatments used in these FRAP experiments. Cells were treated for 4 h with LY294002 (20 μM), AKT VIII (5 μM), or rapamycin (100 nM). As shown in the Western blot of Supplemental Figure S2, AKT phosphorylation at threonine 308 was inhibited after treatment with LY294002 or AKT VIII, so both drug treatments eliminated AKT activation in the PI pathway. Rapamycin eliminated the activating phosphorylation of mTOR at serine 2448 (Supplemental Figure S2).

The region-of-interest photobleaching results reported in Figure 1 averaged together recovery for the pool of EGFP-UAP56 at speckled domains and at adjacent nucleoplasmic sites. When we compared the fluorescence recovery of EGFP-UAP56 in the nucleoplasm with the recovery at speckled domains, we found that the nucleoplasmic and speckled domain fluorescence had tightly bound immobile fractions of 26.5 and 40.5%, respectively. PI3 kinase inhibition decreased the immobile fraction markedly to 7.6% for nucleoplasmic EGFP-UAP56 and 2.8% for UAP56 in speckled domains (Table 1). UAP56 binding in both compartments is similarly affected by inhibition of the PI pathway at PI3 kinase or AKT.

The binding of EJC core proteins is also affected by the PI3 kinase/AKT pathway

UAP56, ALY/REF, and NXF1 are recruited to the 5′ end of an mRNA for export (Cheng et al., 2006) but are also reported to associate with the EJC (Le Hir et al., 2000, 2001). Structural studies of the EJC show it to have a stable core consisting of the proteins eIF4A3, MLN51, and the Y14/MAGO heterodimer (Andersen et al., 2006; Bono et al., 2006). We used FRAP after PI or mTOR pathway inhibition.
PI3 kinase inhibition, suggesting that mTOR did not act entirely through AKT. We propose that both pathways are regulators of mRNA export protein binding at speckled domains.

Because MAGOH interacts with Y14, forming a heterodimer, and Y14 is a putative mRNA export factor (Kataoka et al., 2001), we measured the binding of EGFP-Y14 at speckled domains by FRAP (Supplemental Figure S3 and Supplemental Movie S6). EGFP-Y14 recovered very rapidly at speckled domains ($t_{1/2} = 4.5$ s) with a high immobile fraction of 56%. Drug treatments did not significantly change the kinetics of recovery for the rapidly exchanging fraction. However, after inhibition of PI3 kinase the immobile fraction of EGFP-Y14 was decreased to 43.8% (Figure 3 and Table 2). Inhibition of AKT had greater effect, reducing the tightly bound fraction to 37.3%. Inhibition of mTOR had no significant effect.

Like UAP56, elf4A3 is an ATP-dependent RNA helicase of the DExD/H family (Linder, 2008). A point mutant of elf4A3 that is unable to bind to ATP (Shibuya et al., 2006) was cloned. EGFP-elf4A3 with this K88N point mutation was less concentrated at speckled domains than was the wild-type elf4A3 but was, instead, more widely distributed in the nucleoplasm, similar to the distribution of the UAP56 K95N point mutant, which cannot bind ATP (Kota et al., 2008). EGFP-elf4A3K88N was not, however, excluded from speckled domains, where it recovered after a photobleach with $t_{1/2} = 3.4$ s and an immobile fraction of 16.8%. This decreased binding compared with the wild-type protein (5.3 s, 27.2%) is consistent with decreased speckled domain localization. The tightly bound immobile fraction of EGFP-elf4A3 K88N significantly decreased only after PI3 kinase inhibition with LY294002 to 11.4%. The half-time of photobleach recovery was decreased after PI3 kinase, AKT, or mTOR inhibition from 3.4 s to 2.4, 1.4, and 1.8 s, respectively (Figure 3). This mutant elf4A3, which can still be incorporated in the EJC core complex (Shibuya et al., 2006), retained the ability to be affected by both PI pathway and mTOR pathway signaling.

**Inhibition of the PI3 kinase/AKT pathway increases the export of poly(A) RNA**

Because the inhibition of PI3K, AKT, or mTOR affected the binding of mRNA export–related proteins in live cells, we reasoned that those changes might have consequences on the export of some mRNAs from the nucleus to cytoplasm. We performed a standard mRNA export assay according to Okada et al. (2008) and Wickramasinghe et al. (2010), quantifying nuclear or cytoplasmic poly(A) RNA with biotinylated oligo(dT) and Alexa 488–streptavidin (Figure 4). Inhibition of PI3 kinase with LY294002 or inhibition of AKT with AKT I VIII caused a significant decrease in nuclear poly(A) RNA and a significant increase in cytoplasmic poly(A) RNA without changing the total signal per cell. Using both inhibitors simultaneously caused a larger effect, increasing our confidence that the PI3 kinase/AKT branch of this complicated pathway is affecting mRNA export (Figure 4).
The binding of EJC core proteins, as measured by FRAP, is regulated by PI3 kinase, AKT, and mTOR. HeLa cells were transfected with EGFP-fusion proteins. After 48 h, cells were treated with 20 μM PI3 kinase inhibitor LY294002 (green circles) for 2.5 h, 5 μM AKT inhibitor VIII (blue triangles) for 3.5 h, or 100 nM of the mTOR inhibitor rapamycin (red squares) for 4.5 h. Black squares, untreated cells. Normalized fluorescence recovery curves are presented for the EJC fusion proteins EGFP-eIF4A3, EGFP-MAGOH, and EGFP-Y14. Also shown is EGFP-eIF4A3 K88N, a point mutant that cannot bind ATP. Each colored arrow marks the t½ of recovery for the curve of the same color. Statistical analysis of the differences between averaged FRAP curves was by paired one-tailed t test: untreated EGFP-eIF4A3 vs. LY294002 (p = 0.0002), AKT (p = 0.0002), rapamycin (p = 0.0002); untreated EGFP-MAGOH vs. LY294002 (p = 0.0001), AKT (p = 0.0001), rapamycin (p = 0.0001); untreated EGFP-Y14 vs. LY294002 (p = 0.0001), AKT (p = 0.0001), rapamycin (p = 0.0527). Means are plotted with standard errors for n cells in each treatment group.

The drug treatments did not change the poly(A) distribution within the nucleus, where it was most concentrated in RNA-splicing speckled domains. In the cytoplasm, however, AKT inhibition caused a decreased perinuclear localization of poly(A). This change was not seen after PI3 kinase or mTOR inhibition.

**Inhibition of AKT increases the export of specific endogenous mRNAs**

Measurement of nuclear and cytoplasmic poly(A) levels by fluorescence in situ hybridization (FISH) has been a standard assay for assessing nuclear export of mRNA (Strasser et al., 2002; Okada et al., 2008; Wickramasinghe et al., 2010). There are limitations to this approach; the results present an average nuclear-to-cytoplasmic ratio for a very heterogeneous population that includes many nuclear poly(A) RNA species that are noncoding and not exported (Brown et al., 1992; Rinn et al., 2007; Amaral et al., 2011). For mRNAs the export of different species may be regulated differently and, therefore, differently affected by the experimental manipulation.

For these reasons, we quantified changes in the nuclear-to-cytoplasmic ratio of specific mRNAs by quantitative real-time (qRT)-PCR assay. In initial experiments, drug inhibition of PI3 kinase and AKT treatments changed the nuclear-to-cytoplasmic ratio of several housekeeping gene mRNAs that are commonly used for normalization. Normalization by cell number was an improvement, but there were experiment-to-experiment variations that we attributed to the varying yield in RNA isolation from nuclear and cytoplasmic fractions.

To overcome this problem, we designed an assay with improved quantification for measuring the nuclear and cytoplasmic fractions of individual mRNA species by normalization to a synthetic internal standard RNA added to the nucleus and to the cytoplasm in exactly the same amount before RNA isolation. In vitro–transcribed miniUAA1 mRNA, an RNA not present in human cells, was used as the synthetic internal standard (Wong and Medrano, 2005; Amrani et al., 2008). Normalization to this internal standard corrected for any nuclear-to-cytoplasmic or sample-to-sample variation in protocol steps, including TRIzol mRNA isolation, RNA precipitation, DNase I digestion, and reverse transcription to generate cDNAs. The use of an internal standard also allowed the calculation of real rather than relative nuclear-to-cytoplasmic ratios.

RNA from whole cells, as well as from the separated cytoplasmic and nuclear fractions, each spiked with a constant and known mass of the internal standard, was extracted and isolated before qRT-PCR to quantify individual species. The quality of our separation of nucleus and cytoplasm was measured by RT-PCR. The nuclear

![Table 2: FRAP measurements for proteins of the EJC core complex.](image)

|                | EGFP-eIF4A3 | EGFP-MAGOH | EGFP-Y14 | EGFP-eIF4A3 K88N |
|----------------|-------------|------------|----------|-----------------|
| Untreated      | Recovery half-time (s) | 6.2        | 10.2     | 4.5             | 3.4             |
|                | Immobile fraction (%)   | 55.4 ± 3.0 | 72.0 ± 1.5 | 56.0 ± 2.0   | 16.8 ± 0.8    |
| LY294002       | Recovery half-time (s) | 3.4        | 12.1     | 5.2             | 2.4             |
|                | Immobile fraction (%)   | 36.4 ± 2.5 | 47.7 ± 4.2 | 43.8 ± 3.6   | 11.0 ± 0.7     |
| AKT I VIII     | Recovery half-time (s) | 3.6        | 12.9     | 4.3             | 1.5             |
|                | Immobile fraction (%)   | 33.7 ± 2.9 | 51.2 ± 3.4 | 37.3 ± 2.2   | 17.0 ± 0.6     |
| Rapamycin      | Recovery half-time (s) | 4.1        | 10.9     | 5.6             | 1.8             |
|                | Immobile fraction (%)   | 51.46 ± 2.5 | 42.2 ± 4.0 | 61.4 ± 2.4   | 16.1 ± 0.6     |
Intronless mRNAs are exported efficiently by mechanisms using UAP56, ALY/REF, and NXF1 (Huang et al., 1999, 2003; Huang and Steitz, 2001; Herold et al., 2001; Erkmann et al., 2005; Farny et al., 2008; Taniguchi and Ohno, 2008; Read and Digard, 2010). The nuclear export of intron-containing mRNAs and intronless mRNAs might be differently regulated by signal transduction pathways. Histone mRNAs present a matched set of RNAs suitable for examining splicing-dependent differences. Although most histone mRNAs are intronless, a few are spliced. We performed an analysis of nuclear-to-cytoplasmic partitioning for the intronless histone mRNAs—H2B, H4, and H3A (Figure 6B)—and for the spliced histone mRNAs—H1F0, H1FX, and H3F3 (Figure 6C). Once again drug inhibition of AKT decreased the nuclear-to-cytoplasmic ratio of all histone mRNAs, consistent with an increased rate of nuclear export. The presence of an intron did not change the regulation by AKT. Of interest, the inhibition of PI3 kinase increased the nuclear retention of spliced histone H1F0 and H3F3 mRNAs but did not significantly affect the nuclear export of spliced histone H1F0. The nuclear export of all histone mRNAs was unaffected by rapamycin treatment to inhibit mTOR.

Although the drug treatments were short, we checked to see whether they were able to affect the transcription of the mRNAs used in Figure 6A. There were some modest drug-specific and transcript-specific changes in total cellular levels of these mRNAs (Supplemental Figure S4), but these changes could not account for the differences in nuclear-to-cytoplasmic ratios.

Overexpression of the mRNA splicing factor SRm160 causes the appearance of intron-containing mRNAs in the cytoplasm (McCracken et al., 2002). The PI3 kinase/AKT signaling pathway can change the alternative splicing of fibronectin mRNA (Blaustein et al., 2005). We wondered, therefore, whether the effects on mRNA partitioning seen after AKT inhibition might involve increased export of unspliced mRNA. Quantification of spliced and unspliced GAPDH (Supplemental Figure S5) showed that drug inhibition of AKT inhibition did significantly increase the amount of unspliced mRNA in the cytoplasm. The effect was small, however, and could not account for the enhanced export of GAPDH mRNA. In addition, based on the ΔΔCt values, the ratio of spliced to intron-containing RNA in the cytoplasm was, on average, 13, a level insignificant relative to the difference in partitioning after AKT inhibition (Figure 5A).

mRNAs encoding either endoplasmic reticulum (ER)–targeted or mitochondrial proteins have, respectively, a SSCR or mitochondrial signal coding region (MSCR) that appropriately targets the protein in cells. These RNA elements can be found near the 5′ end of the open reading frame of most mRNAs that encode secreted, membrane-bound, or mitochondrial-targeted proteins, and they encode peptide domains that target the newly translated protein to noncoding RNA 7SK was used as a nuclear marker, and the mitochondrial-encoded and restricted cytochrome B mRNA was used as a cytoplasmic marker. As seen in Figure 5A, there was little contamination of nuclear RNA by cytochrome B mRNA and little contamination of cytoplasmic RNA by the nuclear-restricted 7SK. Drug treatment to inhibit mTOR.

FIGURE 4: Inhibition of PI3 kinase or AKT increases cytoplasmic levels and reduces nuclear levels of poly(A) RNA. HeLa cells were treated for 4 h with 20 μM LY294002, 5 μM AKT inhibitor VIII, or 100 nM rapamycin before FISH. Cells were fixed for 10 min on ice and then hybridized with biotinylated 54-mer oligo(dT). The hybridized probe was detected with Alexa 488–conjugated streptavidin. Cells were counterstained with 4′,6-diamidino-2-phenylindole to identify nuclei. (A) Representative field of the poly(A) distribution in untreated HeLa cells. Scale bar, 20 μm. (B) Bar graph of poly(A) in the nucleus or cytoplasm. Values are plotted as the percentage of the total cellular poly(A). Statistical analysis of the significance of differences between untreated and treated samples is by t test. Means are plotted with SDs. ***p ≤ 0.001.
the ER or mitochondria (Palazzo et al., 2007). These sequences also promote nuclear export of these mRNAs by a novel mechanism that might be less dependent on the TREX complex for export than most mRNAs or even be TREX independent (Palazzo et al., 2007; Cenik et al., 2011). For both SSCR- and MSCR-containing mRNAs the first 69 nucleotides after the start codon tend to be adenine poor, whereas at the protein level both sequences are leucine rich, as well as rich in hydrophobic amino acids (Palazzo et al., 2007; Cenik et al., 2011). A comprehensive genome analysis showed that genes encoding either ER-targeting or mitochondrial proteins tend to lack introns in their 5′ untranslated regions (5′-UTRs) when compared with other protein-coding genes (Cenik et al., 2011). From this analysis a model to explain the alternative export mechanism has been proposed (Cenik et al., 2011). If the first element emerging from RNA polymerase II is an intron, the mRNA will be exported by a splicing-dependent pathway using TREX complex assembly. If, on the other hand, the first element is an SSCR or MSCR, then this mRNA will be exported by the alternative pathway (Cenik et al., 2011).

Because AKT inhibition decreased the nuclear and increased the cytoplasmic fractions of the selected mRNAs coding for cytoplasmic and nuclear proteins (Figure 6), we decided to determine whether this signaling pathway would also regulate the nuclear export of mRNAs encoding either ER-targeted or mitochondrial proteins. For this experiment we selected mRNAs without an intron at their 5′-UTR and examined the mRNA and protein sequences to determine their fit to consensus SSCR and MSCR motifs (Palazzo et al., 2007; Cenik et al., 2011).

The ER-targeted protein mRNAs were calreticulin (CALR, NM_004343.3), histocompatibility (minor) 13 (HM13, NM_178582), and olfactory receptor, family 10, subfamily A, member 3 (OR10A3, NM_001003745) mRNAs, which have 5, 15, and 19 adenines, respectively, in their first 70 nucleotides (Figure 7A). At the level of amino acid sequence, first 25 residues of CALR and OR10A3 are rich in leucine and hydrophobic amino acids. On the other hand, the first 25 residues of HM13 are poor in leucine and hydrophobic amino acids (Figure 7). The drug inhibition of AKT and mTORC1 had no significant effect on the nuclear export of these mRNAs (Figure 7A), which suggests that AKT specifically regulates the nuclear export of only a subset of mRNAs requiring the TREX complex. Surprisingly, inhibition of PI3 kinase slowed the nuclear export of these mRNAs (Figure 7).

The nuclear export of selected nuclear mRNAs that encode mitochondrial proteins was also analyzed. F1 ATP synthase (F1; ATP5B), ferredoxin 1 (FDX1), and fumarate hydratase (FH) were chosen because these mRNAs have few adenines in the first 70 nucleotides (5, 2, and 6, respectively) and because these proteins are rich in hydrophobic amino acids (Figure 7B). A fourth mitochondrial protein mRNA, isocitrate dehydrogenase 3 (NAD+; IDH3B), was chosen because it has 10 adenines in the first 70 nucleotides. The drug inhibition of PI3 kinase, AKT, and mTORC1 had no significant effect on the nuclear export of IDH3B, FDX1, and FH mRNAs (Figure 7B). The inhibition of mTORC1 decreased the nuclear export of ATP5B mRNA (Figure 7B). This profile of signal transduction effects was very different from what was observed for mRNAs lacking both MSCRs and SSCRs (Figure 6).

Nuclear and cytoplasmic mRNA levels are at a steady state determined by the rates of transcription and RNA processing and degradation, as well as of mRNA export to the cytoplasm. Measurements of mRNA export that depend on nuclear-to-cytoplasmic ratios can be confounded by these additional variables. First, we decided to test whether the drug inhibition of PI3 kinase, AKT, or mTOR would affect the degradation rates of the

![FIGURE 5: The separation of nuclear and cytoplasmic RNA. (A) The fidelity of nuclear and cytoplasmic RNA separation was assessed by qRT-PCR quantification of RNAs known to be restricted to the nucleus or to the cytoplasm. The small nuclear RNA 7SK was the control for the nuclear fraction. Cytochrome B mRNA is a mitochondrially encoded transcript and not present in the nucleus, making it a good cytoplasmic RNA marker. C values were normalized using miniUAA1 mRNA from yeast added as an internal standard to each fraction after the separation of nuclei and cytoplasm. There was very little nuclear contamination of cytoplasmic RNA and very little cytoplasmic contamination of nuclear RNA. Treatment of cells with 20 μM PI3 kinase inhibitor LY294002, 5 μM AKT inhibitor VIII, or 100 nM mTOR inhibitor rapamycin did not affect the partitioning of either 7SK RNA or cytochrome B mRNA. Each bar represents the average of two independent experiments in which triplicates were analyzed. Error bars represent the SE of the mean. ns, not significant. (B) The quality of the nuclear–cytoplasmic fractionation was also assessed by Western blotting using the large subunit of RNA polymerase II as a nuclear control and γ-tubulin as a cytoplasmic control. The results confirmed that nuclear and cytoplasmic material was cleanly separated.](image-url)
transcription rates of the candidate mRNAs used in the export assays of Figure 6. We used actinomycin D at a concentration that inhibits RNA polymerases I and II (Trask and Muller, 1988). HeLa cells were treated (or not) with actinomycin D (0.5 μg/ml) for 7 h to inhibit all transcription. Over the last 3 h, cells were also treated with the PI3 kinase, AKT, and mTORC1 inhibitors (Supplemental Figure S6). The rate of mRNA degradation was not affected by drug inhibition of PI3 kinase, AKT, and mTORC1, suggesting that the PI3 kinase/AKT and mTORC1 signaling pathways did not affect the degradation of the candidate mRNAs of Figure 6.

Recovery of transcription after actinomycin D inhibition requires hours (Schluederberg et al., 1971), but recovery from 5,6-dichloro-1-β-ribofuranosyl benzimidazole (DRB), an inhibitor of RNA polymerase II elongation (Sehgal et al., 1976; Bensaude, 2011; Nechaev and Adelman, 2011), is rapid. Methods using recovery from DRB have been developed to measure rates of transcription and transcriptional elongation (Singh and Padgett, 2009).

This method (Singh and Padgett, 2009) was used to reversibly block transcription in HeLa cells and measure transcription rates after a chase to remove DRB. After 100 μM DRB treatment of HeLa cells for 7 h, the recovery of pre-mRNA levels was measured by qRT-PCR using intron–exon primer pairs (Supplemental Figure S7). Drug inhibition of PI3 kinase, AKT, and mTORC1 had some transcript-specific effects on transcription rate. These changes could not, however, account for the changes in nuclear-to-cytoplasmic ratios of the mRNAs in our mRNA export assays (Figure 6). Taken together, our data suggest that the changes in the nuclear-to-cytoplasmic ratio of candidate mRNAs after the drug inhibition AKT were not due to decreases in their transcription rates or increases in their degradation rates. The effects of AKT inhibition on nuclear/cytoplasmic partitioning were due to mRNA export. AKT inhibition increases the rate of mRNA export to the cytoplasm for mRNAs that lack an SSCR or MSCR motif.

DISCUSSION
mRNA export has been considered a constitutive process; a transcript that is spliced and processed would automatically be sent to the cytoplasm. On reflection, it would be surprising if export were the only unregulated step in the life cycle of an mRNA. Export to the cytoplasm, and not processing, may be the rate-limiting step in the delivery of some mRNAs to the cytoplasm; nuclear dwell times of 1–20 min (histone H4 and β-actin, respectively) after processing have been calculated for a few selected mRNA species (Gondran et al., 1999). Some mature mRNA molecules do not leave the nucleus at all (Weil et al., 2000). The combined use of FRAP with the experimental manipulation of signal transduction has great potential for identifying the pathways that regulate the assembly of specific macromolecular
complexes. Using this screening approach, we showed that the inhibition of either PI3 kinase or AKT in live cells changes the binding affinity of mRNA export–related proteins in macromolecular complexes at mRNA-splicing speckled domains. These domains are large enough to serve as practical FRAP targets and are enriched in mRNA export factors and transcripts.

One branch of the phosphatidylinositol signal transduction pathway, downstream from PI3 kinase, operates through AKT (Barnett et al., 2005; Franke, 2008). AKT has been reported to phosphorylate the mRNA export linker protein ALY/REF in vitro (Okada et al., 2008). Point mutants that cannot be AKT phosphorylated inhibit bulk poly(A) export (Okada et al., 2008). In vitro studies show that UAP56 binds to mRNA before recruiting ALY/REF and then NXF1. In our speckled-domain photobleaching experiments (Figure 1), EGFP-tagged versions of UAP56, ALY/REF, and NXF1 had a significant decrease in the tightly bound “immobile” fraction after either PI3 kinase or AKT inhibition. This suggests that the PI3 kinase/AKT pathway increases the fraction of macromolecular complexes in which these proteins are tightly bound at speckled domains. Rapamycin, which inhibits mTOR but not the PI3 kinase pathway, did not have this effect, establishing specificity to this regulation.

A second complex of proteins assembling on a nuclear mRNA is the EJC (Kataoka et al., 2000; Le Hir et al., 2000). Three proteins of the EJC core complex (Tange et al., 2005)—eIF4A3, Y14, and MAGOH—also had a decrease in tight binding at speckled domains after PI3 kinase and/or AKT inhibition. MAGOH showed a larger decrease in its tightly bound immobile fraction after mTOR inhibition. It has been reported that Y14 and MAGOH form a stable heterodimer (Ballut et al., 2005; Le Hir and Seraphin, 2008). However, because the binding kinetics of the two proteins, as measured by FRAP, are different, we conclude that they either must be exchanging within the heterodimer or are not always dimerized (Figure 3).

We used FRAP as a screening technique to connect mRNA export to specific signal transduction pathways. Mechanistically, fluorescence recovery in our experiments is the result of multiple processes. These include the steps of equilibrium binding and diffusion (Lele et al., 2004; Nickerson, 2009): 1) the unbinding of bleached molecules, 2) the unbinding of still-fluorescent molecules outside of the bleach zone, 3) the exchange of these two pools of molecules by diffusion, and 4) the binding of fluorescent molecules in the bleach zone. FRAP recovery for an mRNA transcript might also depend on the movement of a completed and processed mRNA transcript away from the transcription site, with recovery coming from unbleached molecules binding to a new mRNA being transcribed (Ben-Ari et al., 2010; Brody et al., 2011). For screening purposes we used the complex nuclear region around single RNA-splicing speckled domains as the photobleach target in each cell. Although the translocation and splicing of protein-coding genes are enriched at and around these speckled domains (Smith et al., 1999; Shopland et al., 2002), there may be complexes of mRNA export proteins present that are not yet on mRNA and may have unique binding constants. Reported rates of protein diffusion in the nucleus (Kruhlak et al., 2000; Carrero et al., 2003) are much faster than the recoveries we report here. FRAP was...
Previously used to explore the complex mechanisms of transcription and RNA processing at short reporter genes (Ben-Ari et al., 2010; Yunger et al., 2010; Brody et al., 2011). Based on these studies, on measured rates of transcription at endogenous genes (Singh and Padgett, 2009; Wada et al., 2009), and on other studies of transcriptional dynamics (reviewed in Palangat and Larson, 2012), we expect the rates of mRNA elongation, movement away from the bleach zone, and recovery by additional transcription to be slow relative to the photobleach recovery rates we report here. We conclude that the changes we observed in photobleach recovery rates caused by PI3 kinase, AKT, and mTOR inhibition were effects on the binding of mRNA export complex proteins in complexes at transcripts and were not measuring steady-state transcript exchange.

We proceeded with experiments to directly measure the effects that the same drugs had on the nuclear export of specific transcripts—first, by a standard assay (Okada et al., 2008; Wickramasinghe et al., 2010) that evaluates export by measuring FISH localization of poly(A) mRNA in the nucleus and the cytoplasm. Inhibition of either PI3 kinase or AKT caused a significant decrease in the nuclear-to-cytoplasmic ratio of poly(A) RNA without changing total poly(A) levels (Figure 4). Simultaneous inhibition of PI3 kinase and AKT caused a slightly larger and more significant change. One explanation for these changes is that the PI3 kinase/AKT branch of the PI pathway induces the nuclear retention of some poly(A) molecules, whereas inhibition of the pathway increases the kinetics of their export.

When specific mRNA molecules were quantified in the nucleus and cytoplasm after drug treatment, the nuclear-to-cytoplasmic ratio of all species was significantly decreased by inhibition of AKT but not by inhibition of PI3 kinase or mTOR (Figure 6). This effect was seen for a subset of spliced and polyadenylated mRNAs, as well as for mRNAs from intronless genes and mRNAs lacking a poly(A) tail. These results confirmed the global result obtained by measuring poly(A) after AKT inhibition (Figure 4). Decreased transcription combined with decreased cytoplasmic turnover for a specific mRNA could theoretically contribute to reduced nuclear-to-cytoplasmic ratios without changes in total cellular levels. We ruled out this possibility by measuring transcription rates (Supplemental Figure 7) and turnover rates (Supplemental Figure 6) for each of the mRNAs of Figure 6 after drug treatments. After AKT inhibition, there were no significant changes in mRNA turnover, and the changes in transcription rate were transcript specific, variable in direction, and could not account for the altered nuclear/cytoplasmic partitioning. We conclude that the export of a subset of mRNAs from the nucleus to the cytoplasm is regulated by the PI3 kinase/AKT signal transduction pathway.

Not all mRNAs were subject to AKT regulation of nuclear export. mRNAs targeted to the endoplasmic reticulum by an SSR motif or to mitochondria by an MSCR did not have altered export after drug inhibition of the AKT branch of the PI3 kinase pathway (Figure 7).

The differential effects of PI3 kinase versus AKT on the mRNA export of specific mRNAs (Figure 6) might result from the branching of the PI pathway downstream of PI3 kinase. Our experiments measure effects of the AKT branch of the downstream pathway. Mutations in budding yeast that reduce inositol hexakis-phosphate (IP6) production in the alternative branch of the PI pathway compromise mRNA export to the cytoplasm (York et al., 1999). Mutants in pcl1, ipk2, or ipk1—all required to produce IP6—cause defects in mRNA export. IP6 and the Nup42-binding protein Gle1 cooperate to stimulate the ATPase activity of the DEcD/H helicase Dbp5 (Alcazar-Roman et al., 2006; Weirich et al., 2006). Gle1 and IP6-activated Dbp5 remodels the mRNP to promote transit across nuclear pores to the cytoplasm (Tran et al., 2007). These are effects of the PI signaling pathway that are downstream from PI(4,5)P2 but do not involve PI3 kinase or AKT. Both branches of the PI pathway downstream from PI3 kinase affect mRNA export, and these effects may be opposing. A growing body of work has localized many enzymes and inositol lipid intermediates of the PI pathway to sites in the nuclear interior and most often to speckled domains (reviewed in Neri et al., 2002; Irvine, 2003; Martelli et al., 2006; Okada and Ye, 2009; Barlow et al., 2010). These include PI(4,5)P2, the substrate of PI3 kinase (Boronenkov et al., 1998; Osborne et al., 2001; Stallings et al., 2005; Bunce et al., 2006), and several PI3 kinases (Didichenko and Thelen, 2001; Resnick et al., 2005). All three isoforms of AKT are observed in both the nucleus and the cytoplasm (Brazil et al., 2004; Martelli et al., 2006). All share a conserved CRM1-interacting nuclear export signal, suggesting that they can shuttle between nucleus and cytoplasm (Saji et al., 2005). The results we report here do not distinguish effects of the nuclear versus the cytoplasmic PI pathway on mRNA export factor binding or on bulk nuclear poly(A) export. Still, the spatial juxtaposition of mRNA export factors and of the PI signal transduction pathway that regulates their binding and function is unlikely to be a coincidence.

MATERIALS AND METHODS

Constructs

Standard procedures for cloning fluorescent fusion proteins were as previously described (Herbert et al., 2002; Wagner et al., 2003, 2004; Kota et al., 2008). Point mutations were generated using a Stratagene (La Jolla, CA) QuikChange Site-Directed Mutagenesis Kit (Kota et al., 2008).

Transfections and treatments

HeLa cells were grown on coverslips and then transfected with plasmids using Lipofectamine 2000 (Life Technologies, Paisley, United Kingdom) in serum-free medium for 4 h. After 48 h, transfected cells were treated for 4 h with drugs inhibiting PI3 kinase, AKT, or mTOR. PI3 kinase was inhibited with LY294002 (Cayman Chemical, Ann Arbor, MI) at a final concentration of 20 μM (Vlahos et al., 1994). mTOR was inhibited with rapamycin (Calbiochem, San Diego, CA) at a final concentration of 100 nM (Hosoi et al., 1999). AKT inhibitor VIII (Calbiochem) was used to inhibit AKT1, AKT2, and AKT3 at a final concentration of 5 μM. mRNA degradation rates were measured after transcription was inhibited for 7 h using actinomycin D at a final concentration of 0.5 μg/ml. Transcription rates were measured by qRT-PCR using intron-exon primer pairs in cells pretreated with DRB at final concentration of 100 μM and then at time intervals after removal of DRB.

Western blots

Proteins from HeLa cell lysates were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes. Western blots were probed with primary antibodies, including rabbit antibodies to mTOR and phospho-mTOR serine 2448 (both from Millipore, Billerica, MA); rabbit antibodies to AKT and phospho-AKT threonine 308 (both from Cell Signaling, Danvers, MA); or mouse monoclonal antibody to actin (clone 4; ICN/VMP, Solon, OH). Secondary anti-rabbit or anti-mouse horseradish peroxidase–conjugated secondary antibodies were from GE Healthcare (Little Chalfont, United Kingdom), and the detection system used the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Fluorescence recovery after photobleaching

FRAP assays were performed at 37°C as previously described (Wagner et al., 2004; Kota et al., 2008). Leica Confocal Software
(Leica Microsystems, Exton, PA) was used to measure the intensity of fluorescence in the bleached region of interest and in the whole nuclear profile at each time point. We analyzed those data using Excel spreadsheets (Microsoft, Redmond, WA). Any remaining fluorescence in the bleached area after the bleach was normalized to zero. To calculate the relative fluorescence intensity ($I_{rel}$) in the bleached area, we used three alternative equations. The first one (Phair and Misteli, 2000) used the equation $I_{rel,t} = N_{t}/N_{0}$. The second approach used the equation $I_{rel,t} = I_{0}N_{t}/N_{0} - I_{0}N_{pbl}/N_{pbl}$. The third equation is derived from the second one by subtracting background fluorescence values measured outside of the cell from each fluorescence intensity measurement. For all three equations $N_{0}$ is the total nuclear fluorescence before bleaching, $N_{pbl}$ is the total nuclear fluorescence in the first image taken after the bleach, $N_{t}$ is the total nuclear fluorescence at time $t$, $I_{0}$ is the fluorescence in the bleach zone before the bleach, $I_{t}$ is the fluorescence in the bleach zone at time $t$, and $I_{pbl}$ is the fluorescence in the bleach zone in the first image taken after the bleach. Recovery curves were drawn using Prism 5 (GraphPad Software, La Jolla, CA). The percentage of immobile protein (immobile fraction) was determined after normalization of fluorescence in the bleached area to 0 in the first postbleach image and 1 in the prebleached image, and it was calculated as the percentage difference between the relative fluorescence asymptote of the recovery curve and a relative recovery of 1, a value that would reflect complete recovery without an immobile fraction. Individual time points are presented as means, with error bars showing standard errors. The best fit for these photobleach recoveries was obtained using an exponential association curve: $F(t) = F_{max}(1 - e^{-kt})$. All half-times of recovery and immobile fractions were calculated from a best fit to this equation.

Multiple cells were examined by FRAP over a 2-h interval for each drug treatment in each experiment. The times between drug addition and photobleaching are reported as a mean. Three independent experiments were done for each EGFP-fusion protein paired with every drug. The recovery data averaged together in one recovery curve were from the same confocal session. Drug-treated and control cells were always from the same transfection and photobleached in the same confocal session. The first two sets of independent photobleaching experiments were performed on a Leica SP1 laser scanning confocal microscope (Leica, Wetzlar, Germany), and the last set (Figures 1 and 3) was performed on a Leica SP5 AOBS system. Results were independent of the system used. The results presented were from the Leica SP5 AOBS, using a 40×/numerical aperture 1.3 water immersion objective. EGFP fluorescence was photobleached using the 488-nm line of an argon laser for 200 ms at 20 mW. Nuclear speckled domains were spot photobleached. EGFP-NXF1, which was less concentrated in speckled domains, was region-of-interest photobleached.

Fluorescent in situ hybridization

FISH was performed by a modification of Johnson et al. (1991). A 54-mer oligo(dT) (Qiagen, Valencia, CA) was biontinated with a Roche kit (Roche, Indianapolis, IN). HeLa cells were fixed in 4% paraformaldehyde for 10 min on ice and then permeabilized using 0.5% Triton X-100 in CSK buffer for 5 min at room temperature. After dehydration with ethanol to 70% and then 100%, cells were probed overnight the biontinated oligo(dT) at 37°C in the presence of 15% formamide. After several washes in SSC buffer (4×, 2×, and 1×) cells were stained for 3 h (at 37°C) with Alexa 488–conjugated streptavidin in a buffer with 4× SSC buffer and 1% of RNase-free bovine serum albumin (Invitrogen/Life Technologies, Carlsbad, CA).

Nuclear/cytoplasmic fractionation

Nuclear and cytoplasmic fractions were separated by the protocol developed by Lamond and colleagues for proteomic studies (Andersen et al., 2002). Briefly, equal number of HeLa cells (5 × 10⁶) were plated in 15-cm dishes and after 24 h were treated using LY294002 (20 μM), AKT inhibitor VIII (5 μM), or rapamycin (100 nM) for 4 h. After treatment and trypsinization, cells were centrifuged at 1000 rpm for 5 min and rinsed with ice-cold phosphate-buffered saline. Cell pellets were gently resuspended in 1 ml of lysis buffer A + VRC (4 mM 2-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 2 mM vanadylbisulfate complex, pH 7.9) and immediately split into two tubes labeled Total and CN, respectively, and containing 500 μl each. Cells from both tubes were kept on ice for 5 min and broken to release their nuclei with a prechilled Dounce homogenizer (20 gentle strokes with a tight pestle). Dounced cells from the CN tubes were centrifuged at 228 × g for 5 min at 4°C. The supernatant was retained as the cytoplasmic fraction and kept on ice. The nuclear pellet was washed once again with buffer A + VRC, centrifuged, resuspended in 500 μl of buffer S1 + VRC (250 mM sucrose, 10 mM MgCl₂, 2 mM vanadylbisulfate complex), and layered over a 500-μl cushion of buffer S3 + VRC (880 mM sucrose, 0.5 mM MgCl₂, 2 mM vanadylbisulfate complex). Nuclei were then centrifuged at 2800 × g for 10 min at 4°C and resuspended in 500 μl of buffer A + VRC. All buffers were made using water pretreated with 0.1% (vol/vol) diethylpyrocarbonate.

Quantitative real-time PCR

A 120-pg amount of a synthetic, capped, and poly(A)-containing mRNA (miniUAA1; Amrani et al., 2008) was in vitro transcribed, purified, and added to each tube before TRizol (Invitrogen) extraction. This synthetic mRNA worked as an internal standard or calibrator in our qRT-PCR measurement of endogenous mRNAs. Total, cytoplasmic, and nuclear RNA fractions—now in the presence of 120 pg of the synthetic miniUAA1 mRNA—were isolated using TRizol reagent, digested with DNase I recombinant enzyme (Roche), and purified with DNA-free RNA kit (Zymo Research, Irvine, CA). cDNAs were then synthesized using random primers (Roche) and reverse transcription SuperScript III (Invitrogen). GoTaq mix (Promega, Madison, WI) was used to amplify cDNAs.

All curves were normalized by comparative $C_{t}$ (2-add method) (Wong and Medrano, 2005). Briefly, $C_{t}$ values were corrected for the mean $C_{t}$ value of miniUAA1 mRNA as follows: First, raw $C_{t}$ values of GAPDH mRNA ($C_{GAPDH}$) were corrected using Mean $C_{t(miniUAA1)}$, generating a corrected curve for GAPDH mRNA ($C_{GAPDH,C}$). Then $C_{GAPDH,C} = C_{t(miniUAA1)} - C_{GAPDH,C}$. Because an isolation was performed separating cytoplasmic and nuclear fractions, we created two corrected curves: N-$C_{GAPDH,C}$ for the nuclear fraction of GAPDH mRNA and C-$C_{GAPDH,C}$ for the cytoplasmic fraction of GAPDH mRNA. The two curves were corrected by C-Mean $C_{t(miniUAA1)}$ and N-Mean $C_{t(miniUAA1)}$, respectively. Although we added the same amount of the synthetic mRNA miniUAA1 (120 pg) in every fraction, its $C_{t}$ values were slightly different, so an additional correction was performed using C-Mean $C_{t(miniUAA1)} - N$-Mean $C_{t(miniUAA1)}$.

Eventually two corrected set of data were created: the cytoplasmic versus nuclear fold change (C/N), represented by $Z^C = C_{GAPDH,C} - N_{GAPDH,C}$ and the miniUAA1-corrected fold change, represented by $Z^C_{C/M} = C_{t(miniUAA1)} - N_{C/M}$, which must be as close to 1 as possible. Combining both sets of
data, we have the cytoplasmic versus nuclear corrected and normalized fold change, q(C/N):

\[
q(C/N) = \frac{2^{(-C_{GAPDH,C} - N_{C-GAPDH,C})/2} - C_{MeanC,miniUAA1}}{C_{MeanC,miniUAA1}}
\]

The following primers were used for the quantitative PCR: miniUAA1 forward, 5'-GGAGAAAAAGGTTAAGGCGGCC-3', miniUAA1 reverse, 5'-GGGATCTACTGTTAAAACTGTT-3'; GAPDH forward, 5'-GGTTGGCTCTCCTGACTCAAAC-3'; GAPDH reverse, 5'-GTTGCTGATGCCAAATTTGGT-3'; BRG1 forward, 5'-GGGATGAAGACCTC-TGTCG-CCGCT-3'; H1F0 reverse, 5'-GGGATGTTCTCCTCCTTCTCTATCGGGCTC-3'; H1F0 forward, 5'-GAGAAAAGTTAAGTCGACGCCC-3'; H1F0 reverse, 5'-CCTACTGTTGCCAAACAGGAGG-3'.

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