CRISPR-Cas9-mediated knockout of GCN2 reveals a critical role in sensing amino acid deprivation in bovine mammary epithelial cells

A. M. Edick, J. Audette, and S. A. Burgos*

Department of Animal Science, McGill University, Sainte-Anne-de-Bellevue, QC, Canada H9X 3V9

ABSTRACT

The objective of this study was to determine the role of GCN2 in the response to AA deprivation of primary bovine mammary epithelial cells (BMEC). Cells were isolated from the mammary tissue of 2 lactating Holstein cows by enzymatic digestion, expanded, and induced to differentiate for 5 to 7 d. Relative mRNA expression was measured by real-time quantitative PCR. Protein abundance and site-specific phosphorylation were measured by immunoblotting. Knockout of GCN2 in BMEC was accomplished by lentiviral delivery of a targeted single guide RNA and endonuclease Cas9. To investigate the role of GCN2, we treated lactogenic differentiated BMEC with either culture medium lacking Arg, Leu, and Lys combined or lacking only one of the 3 AA of interest, in comparison to a control with a full complement of AA. Activation of GCN2 was inferred by the phosphorylation status of its downstream target eIF2 α Ser51. We found that GCN2 was activated by both the deprivation of Arg, Leu, and Lys combined and of Arg alone, as shown by a 2.73- and 2.82-fold increase in phosphorylated eIF2 α Ser51 after 1 h of deprivation, respectively. In addition, activation of GCN2 as measured by increased phosphorylation of eIF2 α Ser51 during the deprivation of Arg, Leu, and Lys combined and of Arg alone was sustained for up to 8 h of deprivation. Phosphorylated eIF2 α selectively upregulates translation of transcription factor ATF4, among others, during AA deprivation, which then targets genes necessary for restoring AA homeostasis. Therefore, we investigated the expression of ATF4 transcriptional targets, AA enzyme *ASNS* and AA transporters *SLC7A1* and *SLC38A2*. We found that *ASNS* was upregulated in response to combined AA deprivation and by Arg deprivation alone by 3.6- and 4.51-fold, respectively, at 24 h of treatment. We found that *SLC7A1* was upregulated in response to combined AA deprivation and depriva-

tion of Arg alone by 2.0- and 2.36-fold, respectively, at 8 h of treatment. To establish the role of GCN2 (encoded by *EIF2AK4*) in the response to AA deprivation, we ablated GCN2 in BMEC using clustered regularly interspaced short palindromic repeats-Cas9. We showed that BMEC transduced with single guide RNA targeting *EIF2AK4* were not as responsive to combined AA deprivation, compared with BMEC transduced with nontargeting single guide RNA. Taken together, our results demonstrate a critical role for GCN2 in sensing AA deprivation in BMEC.

Key words: gene editing, amino acid, milk synthesis, bovine mammary epithelial cell

INTRODUCTION

Milk and milk products are a source of essential nutrients, including high-quality protein for humans. For instance, dairy products contribute 16% of protein intake by adults in Canada and the United States (Pasiakos et al., 2015; Auclair et al., 2019). Furthermore, intake of dairy protein promotes maintenance of skeletal muscle mass and may contribute to beneficial cardiometabolic health effects (Fekete et al., 2016). However, there are growing concerns regarding environmental N pollution by dairy cattle largely due to their inefficiency in converting dietary N into milk proteins (Huhtanen and Hristov, 2009). Therefore, there is a need to enhance the efficiency of AA use by the mammary gland for synthesis of milk proteins.

Mammary-specific milk proteins, casein and whey, are synthesized exclusively by bovine mammary epithelial cells (BMEC) mainly from blood-borne AA. Therefore, an adequate supply of AA is critical for milk protein production. The prevailing view is that milk protein production is limited by the AA in shortest supply, the so-called first-limiting AA, such that milk protein production can only be increased by its provision. This theory is encoded in current representations of dietary protein requirements systems for dairy cattle (NRC, 2001). However, recent research has demonstrated that the first-limiting AA theory violates several key assumptions required for it to accurately predict

Received April 11, 2020.

Accepted August 17, 2020.

*Corresponding author: sergio.burgos@mcgill.ca

milk protein responses (Appuhamy et al., 2012). Thus, a better understanding of the underlying molecular mechanisms that govern milk protein responses to AA availability in BMEC is needed to provide a sustainable alternative to the present strategy of overfeeding N to meet AA requirements (Arriola Apelo et al., 2014).

Mammalian cells sense and adapt to fluctuations in the intracellular concentrations of AA through 2 key signal transduction pathways: the integrated stress response (**ISR**) and mechanistic target of rapamycin complex 1 (**mTORC1**; Pakos-Zebrucka et al., 2016; Saxton and Sabatini, 2017). During conditions of single AA deprivation, the accumulation of uncharged tRNA activates GCN2, leading to phosphorylation of eIF2 α at Ser51, a critical control point in mRNA translation, which results in general inhibition of protein synthesis (Berlenga et al., 1999). Part of the attenuation of global protein translation is accomplished through suppression of mTORC1, which under nutrient-replete conditions stimulates protein synthesis by phosphorylation-induced changes in the activity of multiple components of the translational machinery including S6K1 and 4E-BP1 (Hara et al., 1998; Wang et al., 1998; Ye et al., 2015). Phosphorylation of eIF2 α by GCN2 promotes the selective translation of ATF4, a transcription factor that induces the expression of genes that enable adaptation to AA scarcity to restore homeostasis (Vattem and Wek, 2004). In the adaptive response to AA deprivation, ATF4 targets genes involved in AA transport including *SLC7A1* and *SLC38A2*, encoding transporters CAT1 and SNAT2, respectively, as well as biosynthetic enzyme *ASNS*, which is involved in asparagine and glutamate synthesis (Kilberg et al., 2009). Evidence indicates that the presence of EAA prevents activation of the ISR in immortalized bovine mammary cells (MAC-T), whereas their removal has the opposite effect (Appuhamy et al., 2011). However, much less is known about the effects of individual AA deprivation on the ISR and the cascade of molecular events that promote adaptation in primary BMEC.

The mammary gland takes up Arg, Leu, and Lys in excess of the amount required for synthesis of milk proteins, directing them toward energy production and synthesis of NEAA (Clark, 1975; Lapierre et al., 2012). Leucine and Arg can also act as signals that convey information about AA sufficiency to mTORC1 (Saxton and Sabatini, 2017; Wolfson and Sabatini, 2017). Previous studies assessing the effect of AA deprivation on milk protein synthesis in mammary tissue and MAC-T cells showed that deprivation of all AA activated the ISR, but only numerical differences were observed for individual AA (Appuhamy et al., 2012). The branched-chain AA, especially Leu and Ile, are known to play a

stimulatory role in protein synthesis in MAC-T cells and mammary tissue through activation of mTORC1 (Appuhamy et al., 2011, 2012). However, the effects of branched chain AA in vivo on the mTORC1 pathway and milk protein yield have differed between studies. For example, Weekes et al. (2006) did not find any change in milk yield or composition, including protein concentration, whereas Doelman et al. (2015) found that a deficiency of all 3 branched-chain AA combined may decrease milk protein yield by preventing mTORC1 upregulation. Lysine is often a limiting AA for milk protein synthesis in dairy cows and has been shown to influence mTORC1 activity and increase the expression of milk protein genes in response to specific Lys ratios in immortalized bovine cells (Manjarin et al., 2014; Nan et al., 2014; Gao et al., 2017).

The clustered regularly interspaced short palindromic repeats (**CRISPR**)-Cas9 is an adaptive microbial immune system first described in *Streptococcus thermophilus*, a bacterial strain commonly used in the production of fermented dairy products (Barrangou et al., 2007). This naturally occurring system was engineered to perform gene editing in mammalian cells and consists of 2 key components: the single guide RNA (**sgRNA**) that designates the target genomic locus and the endonuclease Cas9, which cleaves the targeted DNA. Repair of the double-stranded DNA breaks by the error-prone nonhomologous end joining pathway results in indel mutations that cause frameshifts that prevent the production of a functional protein encoded by that gene. This technology has revolutionized several fields but has yet to be applied to elucidate the molecular mechanisms governing milk protein synthesis in BMEC. We hypothesized that deprivation of individual AA is sensed by GCN2 in BMEC leading to the upregulation of select genes involved in AA homeostasis. To test this hypothesis, we first characterized the phosphorylation of eIF2 α and expression of ISR target genes in response to single AA deprivation. Then, we used CRISPR-Cas9 knockout (**KO**) gene editing to target *EIF2AK4*, the gene encoding GCN2, in primary BMEC to characterize the role of GCN2 in sensing AA deprivation.

MATERIALS AND METHODS

Isolation and Culture of BMEC

Bovine mammary epithelial cells were isolated and cultured as described in Huang et al. (2020). Briefly, mammary gland tissue from lactating Holstein cows was aseptically collected at the time of slaughter from a local abattoir and placed into 50-mL centrifuge tubes containing ice-cold Ham's F12 medium supplemented

with $1 \times$ antibiotics/antimycotics (100 U/mL penicillin, 100 μ g/mL streptomycin, 0.25 μ g/mL amphotericin B, and 50 μ g/mL gentamicin) for transport to the laboratory. After removal of visible fat, connective tissue, and blood vessels, mammary tissue was minced into $\sim 1\text{-mm}^3$ pieces using scalpels and then rinsed $5\times$ using ice-cold Ham's F12 medium supplemented with $1 \times$ antibiotics/antimycotics to remove residual milk and blood. The minced tissue was enzymatically digested in Dulbecco's modified Eagle's medium (DMEM)/F12 containing 300 U/mL type-3 collagenase (Worthington Biochemical Corp., Lakewood, NJ), 400 U/mL of hyaluronidase, and 1 mg/mL DNase I supplemented with $1 \times$ antibiotics/antimycotics at 37°C with constant shaking (80 rpm) for 4 h. The tissue digest was filtered through a $200\text{-}\mu\text{m}$ mesh sieve and then centrifuged at $80 \times g$ at room temperature for 30 s. The resulting pellet was highly enriched in mammary epithelial organoids (acini). The pellet was resuspended in BMEC growth medium for outgrowth of mammary epithelial cells. The BMEC growth medium was composed of 1:1 DMEM/F12:MCDB170 (catalog no. M2162, US Biological, Salem, MA), 0.25% (vol/vol) fetal bovine serum, 0.1% (wt/vol) Albumax II, 7.5 μ g/mL bovine insulin, 0.3 μ g/mL hydrocortisone, 5 ng/mL recombinant human epidermal growth factor, 2.5 μ g/mL bovine apo-transferrin, 5 μM isoproterenol, 5 pM 3,3',5-triiodo-L-thyronine, 0.5 pM β -estradiol, 0.1 nM oxytocin, and $1 \times$ antibiotics/antimycotics. The medium was originally developed by Garbe et al. (2009) for selective growth of human mammary epithelial cells under serum-reduced conditions. Primary mammary epithelial cells from acini outgrowths were passaged once for expansion and then cryopreserved. Cell culture reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada) or Thermo Fisher Scientific (Burlington, ON, Canada), unless specified otherwise.

Design and Cloning of Lentiviral Vector for CRISPR Knockout

Single guide RNA targeting exons 1 and 4 of *EIF2AK4* (National Center for Biotechnology Information Gene ID: 513829) in *Bos taurus* genome (Btau 5.0) were designed using CHOPCHOP (<http://chopchop.cbu.uib.no/>; Labun et al., 2016). We selected sgRNA with the highest predicted efficiency scores (Doench et al., 2016), lowest predicted off-targets (up to 3 mismatches in the protospacer), 40 to 60% GC content, and ≤ 1 self-complementarity score. Two control sgRNA were selected from a universal nontargeting (NT) control sgRNA library (Doench et al., 2016). A BLASTn search confirmed that the selected NT sgRNA lacked

sequence homology with the bovine genome (Boratyn et al., 2012). The sequences of the sgRNA used in this study are in Table 1.

The sgRNA were synthesized as single-stranded DNA oligonucleotides by Thermo Fisher Scientific. The 24-bp forward and reverse oligonucleotides including the 20-bp target sequence and BsmBI cohesive end were annealed and then cloned into the lentiCRISPR_V2 vector (Sanjana et al., 2014), a gift from Feng Zhang (52961, Addgene, Watertown, MA), according to the method of Shalem et al. (2014).

To produce lentivirus, HEK293T cells (Q401, GenHunter, Nashville, TN) seeded in 100-mm plates were transfected with LentiCRISPR_v2 plasmid cloned with either NT or *EIF2AK4*-targeting sgRNA, psPAX2 (12260, Addgene), and pMD2.G (12259, Addgene), a gift from Didier Trono, using transfection-grade polyethylenimine 'Max' (MW 40000; 24765, Polysciences, Warrington, PA), in accordance with the manufacturer's instructions. After 24 h of incubation, the medium containing lentiviral particles was harvested, centrifuged at $800 \times g$ for 5 min at room temperature to remove any cells and debris, and stored at -80°C .

Viral Transduction and Antibiotic Selection

First-passage BMEC seeded into 60-mm collagen-coated plates and grown to 50% confluence in antibiotic-free BMEC growth medium were transduced with lentivirus in the presence of 8 μ g/mL polybrene (TR-1003-G, Sigma-Aldrich) and then cultured for an additional 24 h. After 2 d, transduced BMEC were passaged and seeded into 60-mm collagen-coated plates. For selection of stably transduced cells, BMEC were subcultured in growth medium containing 8 μ g/mL of puromycin (P8833, Sigma-Aldrich), which was replaced every 2 d. Transduced BMEC were grown to 50 to 60% confluence for collection and cryopreservation.

Genomic Cleavage Assay

The GeneArt Genomic cleavage assay detection kit (A24372, Thermo Fisher Scientific) was used to determine the efficiency of genomic cleavage at the *EIF2AK4* locus in stably transduced BMEC according to the manufacturer's instructions (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/A24372_GeneArt_GenomicCleavage_Detect_Kit_man.pdf). The sequences of the primers used for PCR amplification of the genomic region flanking each sgRNA are in Table 1. The cleavage efficiencies were calculated according to the manufacturer's instructions as gene modification efficiency = $1 - [(1 - \text{fraction cleaved})^{1/2}]$.

Table 1. List of single guide RNA (sgRNA) used in this study

sgRNA	Sequence (5'→3')	Forward primer (5'→3')	Reverse primer (5'→3')	Exon	Product size (bp)
<i>EIF2AK4_1</i>	CACCGCACTGCTATGGCCGG	TAGTCTTCCTGCGCAGAGGGA	GACCAAGACATGCCAAGTTCC	1	436
<i>EIF2AK4_2</i>	GGAGGCAGTTAGAGGCCAAG	TTCTGCTGTGTTACCTCC	TCTAGGGGCTCTTTTCACA	4	476

Cell Treatments

For experiments, second passage BMEC were seeded into collagen-coated plates and grown to near confluence. For experiments involving stably transduced cells, the medium contained 4 $\mu\text{g/mL}$ puromycin. The medium was changed every 2 d. To induce lactogenic differentiation, cells were incubated in DMEM modified to contain 3.5 mM D-glucose and 1 mM sodium acetate supplemented with lactogenic hormones (5 $\mu\text{g/mL}$ each of bovine insulin, ovine prolactin, and hydrocortisone), 5 $\mu\text{g/mL}$ bovine apo-transferrin, 0.5 mg/mL BSA, and $1 \times$ antibiotic-antimycotics for 5 to 7 d. The concentrations of AA in DMEM was (in μM): Cys, 200; Gln, 4,000; Gly, 400; His, 200; Ile, 800; Met, 200; Phe, 400; Ser, 400; Thr, 800; Trp, 80; Tyr, 400; and Val, 800. Cells were incubated in low-glucose DMEM without Arg, Leu, Lys (D9443, Sigma-Aldrich) for deprivation of all 3 AA. For treatments lacking either Arg, Leu, or Lys alone, the appropriate AA were reconstituted in Arg-, Leu-, Lys-free DMEM using cell culture-grade AA purchased from Sigma-Aldrich. Lactogenic differentiated BMEC were treated with either control medium containing all AA or medium lacking either Arg, Leu, or Lys alone or all 3 AA for either 1 h for acute experiments or 1, 4, 8, and 24 h for time course experiments, as specified in the figure legends.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from cells using the TRI-Reagent (Sigma-Aldrich), according to the manufacturer's instructions (<https://www.Sigma-Aldrich.com/technical-documents/protocols/biology/tri-reagent.html>). The RNA yield and purity were quantified by measuring absorbance at 260 and 260/280 nm, respectively, using a Take-3 micro-volume plate in an Epoch microplate spectrophotometer (BioTek, Winooski, VT). The RNA integrity was measured as the ratio of 28S to 18S rRNA subunits by bleach agarose gel electrophoresis, as described in Aranda et al. (2012). Total RNA (1 μg) was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad Laboratories) in a T100 thermal cycler (Bio-Rad Laboratories), according to the manufacturer's instructions (<https://www.bio-rad.com/webroot/web/pdf/lsr/literature/4106228.pdf>).

Quantitative real-time PCR (qPCR) was performed using SsoAdvanced Universal SYBR green (Bio-Rad Laboratories) and 0.5 μM gene-specific primers in a 10- μL reaction containing 100 ng of cDNA on a CFX96 Touch Real Time PCR System (Bio-Rad Laboratories). Gene-specific primers were designed using Primer-BLAST (Ye et al., 2012). Primers used for qPCR in this study are in Table 2. For each primer pair, we

determined the optimal annealing temperature, performed melting curve analyses, determined amplicon length by agarose gel electrophoresis, and tested the efficiency of the qPCR reaction using a 4-fold serial dilution curve according to the method of Taylor et al. (2019). Relative gene expression was calculated according to the $\Delta\Delta C_q$ method using the geometric mean of 3 reference genes (*GAPDH*, *ACTB*, and *PPIA*) in the CFX Maestro Software (Bio-Rad Laboratories).

Immunoblotting

Cells were rinsed twice with ice-cold PBS before lysis in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (vol/vol) Triton X-100, 1 mM EDTA, 50 mM β -glycerophosphate, 50 mM NaF, 10 mM Na_4PO_7 , 10 mM Na_3VO_4 , and supplemented with a protease inhibitor cocktail (Sigma-Aldrich) on ice for 15 min. The cell lysates were centrifuged at $15,000 \times g$, 4°C , for 15 min. A portion of the cleared cell lysate was combined with $5 \times$ SDS loading buffer and incubated at 95°C for 5 min. Another portion was used to measure protein concentration using a BCA protein assay kit (Thermo Fisher Scientific) with BSA as standard. Equal amounts of protein were resolved by SDS-PAGE and then transferred onto polyvinylidene fluoride membranes. The membranes were blocked in 5% (wt/vol) nonfat milk in Tris-buffered saline containing 0.1% (vol/vol) Tween 20 (TBS-T) at room temperature for 1 h and then incubated with primary antibodies raised against phospho-specific proteins diluted in 5% nonfat milk in TBS-T at 4°C with constant rocking overnight. Primary monoclonal antibodies (clone name; catalog number) used were phospho-eIF2 α Ser51 (D9G8; 3398), eIF2 α (D7D3; 5324), phospho-p70 S6 kinase Thr389 (1A5; 9206), p70 S6 kinase (49D7; 2708), and α -tubulin (11H10; 2125) were obtained from Cell Signaling Technologies (Danvers, MA). The GCN2 polyclonal antibody (300–555A-T) was from Bethyl Laboratories (Montgomery, TX). After washing $6 \times$ in TBS-T for 5 min, membranes were incubated with secondary antibodies diluted 1:10,000 in 5% nonfat milk in TBS-T at room temperature for 1 h with constant shaking. After washing in TBS-T, the bound horseradish peroxidase-linked secondary antibodies were visualized by chemiluminescence (Bio-Rad Laboratories). The signal intensity was quantified using the Image Lab Software (Bio-Rad Laboratories). After detection of the phospho-specific signal, the antibodies were stripped-off the membranes by incubation in 62.5 mM Tris-HCl, pH 6.8, 2% (wt/vol) SDS, and 100 mM 2-mercaptoethanol at 50°C for 30 min with constant rocking. The membranes were washed, blocked, and re-probed with primary antibodies that recognized the proteins irrespective of their phosphorylation state.

Table 2. List of primers used in this study

Gene symbol	mRNA accession no.	Forward primer (5'→3')	Reverse primer (5'→3')	Primer start position	Exon junction	Product size (bp)
<i>ACTB</i>	NM_173979.3	GACCCAGATCATGTTCCGAGA	CTCATAGATGGGCACCGTGT	449, 593	455/456	145
<i>ASNS</i>	NM_001075653.1	TATCCAGAGAGAGCCTGGAGC	GGACCCCTGTGTGCAATCTT	106, 223	112/113	118
<i>GAPDH</i>	NM_001034034.2	GAGGGCTGCCAGAAATATC	CCAGTGAGCTTCCCGTTGAG	664, 754	742/743	91
<i>PPIA</i>	NM_178320.2	GGCAAGTCCATCTATGGCGA	GCCATCCAAACCACTCAGTCT	234, 383	373/374	150
<i>SLC7A1</i>	NM_001135792.1	GGTCTTACGATACCAAGCCCG	GTCGTGAGATCGCTGCTGCT	1,284, 1,397	1,292/1,293	114
<i>SLC38A2</i>	NM_001082424.1	TGAAGAGCTTAAAGCCGCA	GGTATCCAAAGAGGGCAGCA	1,290, 1,393	1,304/1,305	104

The signal intensity values for phospho-specific and total proteins were first normalized to the loading control, α -tubulin. Then, to normalize the signals across experiments, each normalized phosphorylated-to-total protein ratio was divided by the sum of the ratios for that biological replicate according to the method described by Degasperis et al. (2014). Site-specific protein phosphorylation is expressed as arbitrary units.

Statistical Analyses

Results are from experiments repeated 3 separate times, each with BMEC derived from 2 independent cows, unless otherwise stated. Data were analyzed using PROC MIXED in the SAS/STAT software version 9.4 (SAS Institute Inc., Cary, NC). For AA deprivation experiments, data were analyzed according to a randomized complete block design. The model included the fixed effect of treatment, the random effect of donor cow, and the error term. Treatment means were separated post hoc using Dunnett's test. For genomic editing experiments, data were analyzed according to a split-plot design where the donor cow was the whole plot and the sgRNA within donor cows were the subplots. The model included the fixed effects of treatment, sgRNA, and sgRNA by treatment interaction, the random effects of donor cow, the sgRNA within donor cow, and the error term. The method of Kenward-Roger was used to estimate degrees of freedom. Data are presented as least squares means $\pm$ standard error. A P -value of <0.05 was considered significant.

RESULTS

Effect of Individual AA Deprivation on ISR and mTORC1 Signaling

To evaluate the effect of acute AA deprivation on ISR and mTORC1 signaling, we incubated lactogenic differentiated BMEC in medium lacking Arg, Leu, and Lys (**3AA**) for 1 h and measured the phosphorylation of GCN2 and mTORC1 targets eIF2 α Ser51 and S6K1 Thr389, respectively. Compared with cells incubated in control medium containing all AA, combined deprivation of Arg, Leu, and Lys activated GCN2, as inferred by eIF2 α Ser51 phosphorylation, which increased by 2.73-fold ($P < 0.001$; Figure 1A). In addition, deprivation of Arg, Leu, and Lys decreased phosphorylation of S6K1 Thr389 by 75% ($P < 0.001$; Figure 1B).

We then tested the effects of the individual AA on phosphorylation of eIF2 α Ser51 and S6K1 Thr389. Deprivation of Arg and Lys, but not Leu, induced eIF2 α Ser51 phosphorylation ($P < 0.001$) by 2.82- and 2.08-fold, respectively (Figure 1C). Only deprivation of Arg

affected S6K1 phosphorylation, decreasing it by 70% ($P < 0.001$; Figure 1D). Taken together, these results indicate that of the individual AA tested, deprivation of Arg alone has the greatest effect on the ISR and mTORC1 pathways.

Time Course of AA Deprivation on ISR and mTORC1

To assess the effects of chronic AA deprivation on the ISR and mTORC1 pathways, we incubated differentiated BMEC in DMEM lacking Arg, Leu, and Lys combined or Arg alone for up to 24 h. We found that combined deprivation of Arg, Leu, and Lys or Arg alone increased eIF2 α Ser51 phosphorylation by at least 2-fold at 1, 4, and 8 h ($P < 0.001$), but not at 24 h (Figure 2A), compared with cells incubated in medium containing the full complement of AA. Furthermore, combined deprivation of 3AA or Arg alone diminished mTORC1 activity at 1 and 4 h but not thereafter (Figure 2B). Combined deprivation of 3AA and Arg alone for 1 h decreased S6K1 Thr389 phosphorylation by 41% and 25%, respectively ($P = 0.039$). At 4 h, combined deprivation of 3AA decreased S6K1 Thr389 phosphorylation by 13% ($P = 0.045$) and Arg deprivation increased S6K1 Thr389 phosphorylation by 25% ($P = 0.045$).

Time Course of AA Deprivation on ATF4 Transcriptional Targets

To evaluate the effects of AA deprivation on the downstream targets of the ISR, we measured the expression of ATF4 transcriptional targets in BMEC deprived of Arg, Leu, and Lys or Arg alone for 1, 4, 8, and 24 h. Compared with cells incubated in medium containing all AA, deprivation of 3AA and Arg alone for 24 h induced *ASNS* expression by 3.6- and 4.51-fold ($P = 0.014$), respectively (Figure 3). Expression of *ASNS* was also higher at the 8-h time point in response to both 3AA (2.41-fold) and Arg deprivation (2.66-fold), albeit not significantly ($P = 0.055$). Incubation in medium lacking 3AA or Arg alone also induced *SLC7A1* expression at 8 h by 2.0- and 2.36-fold ($P = 0.003$), respectively (Figure 3). The expression of *SLC38A2* was not different among treatments.

CRISPR-Cas9-Mediated Knockout of GCN2 in BMEC

To characterize the role of ISR on AA in primary BMEC we used CRISPR-Cas9 KO technology to ablate the GCN2 protein. To this end, we designed 2 independent sgRNA targeting exon 1 and 4 of *EIF2AK4* (Figure 4A). We confirmed that BMEC transduced with sgRNA *EIF2AK4*-1 and *EIF2AK4*-2 cleaved the DNA

at their respective loci when compared with NT sgRNA (Figure 4B). Furthermore, GCN2, the protein encoded by *EIF2AK4*, was completely ablated as no protein was detectable in BMEC transduced with sgRNA targeting the gene compared with NT sgRNA (Figure 4C).

Effect of AA Deprivation on ISR and mTORC1 Signaling in GCN2 Knockout BMEC

Once our genetic model of GCN2 KO in BMEC was established, we evaluated the effects on the ability of BMEC to activate the ISR in response to AA depriva-

tion of Arg, Leu, and Lys combined. We found that BMEC transduced with NT sgRNA were responsive to the AA deprivation, showing a 2.57-fold increase in eIF2 α Ser51 phosphorylation ($P = 0.031$). In contrast, those transduced with sgRNA targeting *EIF2AK4* showed no response, indicating decreased sensitivity to AA deprivation through the ISR (Figure 5A). Amino acid signaling through mTORC1 was unaffected by GCN2 ablation, as AA deprivation decreased phosphorylation of S6K1 Thr389 in cells infected with NT by 71% ($P < 0.001$), *EIF2AK4*-1 by 61% ($P < 0.001$), and *EIF2AK4*-2 by 33% ($P = 0.037$; Figure 5B).

Figure 1. Effect of individual AA deprivation on integrated stress response and mechanistic target of rapamycin complex 1 signaling in bovine mammary epithelial cells (BMEC). Lactogenic differentiated BMEC were incubated in complete medium (Ctrl) or deprived of Arg, Leu, and Lys combined (-3AA; A and B) or alone (C and D) for 1 h. Phosphorylation (p) of (A and C) eIF2 α Ser51 and (B and D) S6K1 Thr389 was measured by immunoblotting. Signal intensities of phosphorylated and total protein levels were normalized to α -tubulin (loading control). The signal intensity of phospho-specific proteins was divided by the total protein levels and then normalized across experiments, expressed as arbitrary units. Values are LSM $\pm$ SE for $n = 5$ or 6 . *** $P < 0.001$. Representative immunoblots are shown.

DISCUSSION

Previous studies have shown that intracellular AA concentrations can influence the ISR and mTORC1 signal transduction pathways leading to changes in cellular and milk protein synthesis in mammary immortal-

Figure 2. Time course of AA deprivation on integrated stress response and mechanistic target of rapamycin complex 1 bovine mammary epithelial cells (BMEC). Lactogenic differentiated BMEC were incubated in complete medium (Ctrl) or deprived of Arg, Leu, and Lys combined (-3AA) or Arg alone for 1, 4, 8, and 24 h. Phosphorylation (p) of (A) eIF2 α Ser51 and (B) S6K1 Thr389 was measured by immunoblotting. Signal intensities of phosphorylated and total protein levels were normalized to α -tubulin (loading control). The signal intensity of phospho-specific proteins was divided by the total protein levels and then normalized across experiments, expressed as arbitrary units. Values are LSM $\pm$ SE for $n = 3$. *** $P < 0.001$; * $P < 0.05$. Representative immunoblots are shown.

Figure 3. Time course of AA deprivation on downstream targets of the ATF4 bovine mammary epithelial cells (BMEC). Lactogenic differentiated BMEC were incubated in complete medium (Ctrl) or deprived of Arg, Leu, and Lys combined (-3AA) or Arg alone for 1, 4, 8, and 24 h. mRNA abundance of (A) *ASNS*, (B) *SLC7A1*, and (C) *SLC38A2* was measured by quantitative real-time PCR. The abundance of target mRNA is expressed as the normalized fold change ($\Delta\Delta C_q$). Values are LSM $\pm$ SE for $n = 3$. ** $P < 0.01$; * $P < 0.05$; † $P < 0.10$.

Figure 4. Clustered regularly interspaced short palindromic repeats-Cas9 mediated knockout of GCN2 in bovine mammary epithelial cells (BMEC). (A) A schematic diagram of *EIF2AK4* exons (numbered boxes) in chromosome (Chr.) 10 targeted by the single guide RNA (sgRNA). The sequence targeted by each sgRNA is shown with the protospacer adjacent motif underlined. The relative position of the primer pairs (F1-R1 and F2-R2) for genomic cleavage is shown. (B) Genomic cleavage assay to detect endogenous target cleavage that resulted in indel mutations. Cleavage efficiency was calculated using the fraction of cleaved to uncleaved DNA. The genomic cleavage assay employs the T7 endonuclease I (T7EI) to detect DNA cleavage present in input samples of BMEC transduced with nontargeting control sgRNA (NT), or sgRNA targeting coding region of *EIF2AK4* (*EIF2AK4-1* and *EIF2AK4-2*). (C) Protein abundance of GCN2 (encoded by *EIF2AK4*) and α -tubulin (loading control) was measured by immunoblotting. Representative immunoblots are shown.

ized cells and tissue slices (Appuhamy et al., 2012; Ariola Apelo et al., 2014). Appuhamy et al. (2012) found that no single AA influenced eIF2 α phosphorylation in mammary tissue and MAC-T cells. However, there is a

Figure 5. Effect of AA deprivation on the integrated stress response and mechanistic target of rapamycin complex 1 pathways in GCN2 knockout bovine mammary epithelial cells (BMEC). Lactogenic differentiated GCN2 knockout BMEC transduced with nontargeting (NT) control single guide RNA (sgRNA) or sgRNA targeting the coding region of *EIF2AK4* (*EIF2AK4-1* and *EIF2AK4-2*) were deprived of Arg, Leu, and Lys combined (-3AA) or incubated in complete medium (Ctrl) for 1 h. Phosphorylation (p) of (A) eIF2 α Ser51 and (B) S6K1 Thr389 was measured by immunoblotting. Signal intensities of phosphorylated and total protein levels were normalized to α -tubulin (loading control). The signal intensity of phospho-specific proteins was divided by the total protein levels and then normalized across experiments, expressed as arbitrary units. Values are LSM $\pm$ SE for $n = 5$ or 6 . *** $P < 0.001$; * $P < 0.05$. Representative immunoblots are shown.

time-dependent nature to both the ISR and mTORC1 pathway response to AA in BMEC, MAC-T cells, and mammary tissue slices, showing activity minutes to hours after changes in AA availability (Moshel et al., 2006; Burgos et al., 2010; Appuhamy et al., 2011). This led us to investigate the ISR signaling cascade over a 24-h period. We found that both the combined deprivation of AA and the individual deprivation of Arg acutely activated eIF2 α through the ISR sensor GCN2. In addition, the ISR shares an endoplasmic reticulum stress sensor with the unfolded protein response, PERK. Just as GCN2 phosphorylates eIF2 α during AA deprivation, PERK phosphorylates eIF2 α in response to ER stress. Nichols et al. (2017) recently found that expression of some components of the unfolded protein response in the mammary gland specific to the PERK arm were suppressed during EAA infusion into the abomasum of lactating Holstein cows, which led to an increase in milk protein yield. In this same study, they also found that mTORC1 in the skeletal muscle responds to abomassally infused EAA, whereas nutritional regulation in response to EAA through mTORC1 was not apparent in the mammary gland. In fact, several studies suggest that a mechanism other than, or in addition to, the mTORC1 pathway may be responsible for increased milk protein production in response to specific EAA in vivo (Doelman et al., 2015; Nichols et al., 2017). Differences in experimental models and metabolic activity of tissues may influence the ability to capture transient changes in phosphorylation state of signaling proteins. For instance, primary mammary cells and tissues slices can be treated for short periods and collected rapidly, whereas mammary biopsies are typically collected after lengthy infusion periods and take longer to obtain.

The adaptive response to AA deprivation begins when GCN2 kinase recognizes uncharged tRNA; it then phosphorylates eIF2 α , which specifically upregulates ATF4 translation. The ATF4 induces the expression of a gene program necessary for cellular adaptation to the AA deprivation that includes AA transporters and enzymes that help maintain a normal balance of AA in cells. We found that combined deprivation of Arg, Leu, and Lys or Arg alone induced the expression of ATF4 transcriptional targets: AA transporter *SLC7A1* and enzyme *ASNS*. Baumrucker (1984) described the presence of a saturable and sodium-independent transport system responsible for the uptake of Arg and Lys into bovine mammary tissue, which is consistent with the functional characteristics of CAT1, encoded by *SLC7A1* (Verrey et al., 2004). Curiously, deprivation of Arg had a greater effect on *SLC7A1* expression than Arg, Leu, and Lys combined at 8 h. Likewise, at 1 h of deprivation eIF2 α phosphorylation was higher with the deprivation of Arg alone than with combined AA deprivation.

This result was unexpected as GCN2 senses AA status through a surrogate molecule, uncharged tRNA, and thus would be expected that the absence of any one AA would result in an increase in uncharged tRNA and elicit an adaptive response. This indicates that Arg may have a unique role in signaling the deprivation of AA, although further investigation is required. Contrary to our findings, a study on MAC-T cells showed that Arg supplementation upregulated genes involved in mRNA translation and AA transport, including *SLC38A2* and *SLC7A1*, which encode for SNAT2 and CAT1, respectively (Salama et al., 2019). SNAT2 is responsible for the transport of small neutral AA, primarily Ala and Gln (Mackenzie and Erickson, 2004). These differences may be due to the use of MAC-T cells, as they are not the most representative BMEC cellular model, according to Jedrzejczak and Szatkowska (2014). These differences may also stem from the fact that in the study by Salama et al. (2019), Arg supplementation was given at twice the normal amount found in DMEM; the amount found in DMEM is considered ideal according to Dong et al. (2018). Furthermore, *SLC7A1* expression in response to the addition of Arg was measured at 6 h (Salama et al., 2019), whereas we did not see the response of AA transporter expression to Arg deprivation until 8 h. A combination of these factors may explain why this recent study on the effects of Arg addition does not mirror our results.

A surprising finding from our study was that Arg demonstrated an increased ability to activate the ISR when compared with the combined deprivation of AA or with other individual AA. Although Arg can be synthesized de novo, dairy cows cannot produce adequate amounts to meet metabolic demands, such that it is considered essential to the diet (NRC, 2001). Studies on mammary AA uptake by arteriovenous difference demonstrated that Arg, Leu, and Lys are taken up in excess of requirements for milk protein synthesis (Clark, 1975). In contrast, uptake of Pro by the mammary gland is not sufficient to support casein synthesis (Clark et al., 1975), but can be synthesized from uptake of precursor AA in excess, such as Arg and Orn (Mepharm, 1982). Proline synthesis begins with the hydrolysis of Arg to urea and Orn by arginase present in the bovine mammary gland, possibly in a dose-dependent manner (Basch et al., 1997). Thus, Arg supply is important for the biosynthesis of Pro to be available in adequate quantities to meet requirements for casein synthesis. This may also offer an explanation for our findings that Arg deprivation had increased potency in eliciting a GCN2-mediated response in comparison with Leu and Lys. Furthermore, there is evidence that Arg supplementation recovered milk fat and protein synthesis in vitro by inhibiting GCN2 activation in BMEC, showing

that an inverse experimental design produced findings that mirror our study (Xia et al., 2016). Likewise, a study focused on the effects of Arg on inflammatory responses in BMEC found that Arg not only reduced the inflammatory response but also upregulated mTOR gene expression and β -CN synthesis (Wu et al., 2016). The ability of Arg to stimulate mTORC1 signaling may be explained by its capacity to bind to upstream sensor and regulator CASTOR1 and lysosomal sensor SLC38A9, another point of regulation for protein synthesis (Wolfson and Sabatini, 2017). Collectively, this suggests that Arg may play an important role in regulating milk protein synthesis in BMEC. Nevertheless, the physiological relevance of these findings may be tempered by the study of Doepel and Lapierre (2011), who showed that deletion of Arg from an abomasal infusion, when all other AA were present, did not affect milk protein yield. However, because estimates of Arg requirements for dairy cows are not well defined, it could be that the supply of MP was above average levels and thus cows were not Arg deficient.

Previous studies have shown that deprivation of Leu decreased S6K1 phosphorylation, albeit less so than total AA deprivation in L-1 immortalized bovine mammary cells and mammary tissue slices (Moshel et al., 2006; Appuhamy et al., 2012). Arginine did not show the same effects of S6K1 phosphorylation (Appuhamy et al., 2012). This contrasts with our findings that deprivation of Arg, but neither Leu nor Lys, affected S6K1 phosphorylation. Furthermore, a study by Gao et al. (2017) showed that the addition of Leu alone stimulated S6K1 phosphorylation in comparison to medium lacking AA in immortalized BMEC. The lack of response to Leu deprivation that we observed was not expected and the reason for this remains unclear.

Lysine deprivation also had a positive effect on eIF2 α phosphorylation, which is in line with the findings of Lin et al. (2018) that Lys promoted protein synthesis in BMEC, as its presence activated global protein synthesis through the mTORC1 pathway. In contrast, Doelman et al. (2015) showed that mammary abundance of phosphorylated eIF2 α was not affected in lactating cows given an abomasal infusion lacking Lys, compared with a complete AA mix. However, we did not find that Lys deprivation significantly affected mTORC1 signaling. This could be due to the difference in the cellular model used to conduct the research, as we used primary BMEC which are a more biologically relevant model (Jedrzejczak and Szatkowska, 2014), instead of a cell line.

We did not observe gross differences in growth rate between BMEC transduced with NT and *EIF2AK4* sgRNA. The GCN2 KO mice are viable, fertile, and exhibit no phenotypic abnormalities when reared under

standard growth conditions; however, their ability to develop normally was hindered during AA deprivation (Zhang et al., 2002). This study highlights that the difference between KO and wild-type mice are only noticeable during conditions of AA deprivation (i.e., when GCN2 activity would normally be elevated to restore AA homeostasis). On that note, during the relatively short duration of the AA deprivation on differentiated GCN2 KO BMEC in our study, we did not observe any differences in proliferation in the treated cells either. We speculate that the effect of AA deprivation on GCN2 KO BMEC proliferation would be more apparent if the duration of AA deprivation was extended. Future studies should expand on the role of GCN2 and downstream eIF2 α phosphorylation in the cellular adaptation to AA deprivation including its effect on milk proteins, particularly under prolonged deprivation and for other key AA such as methionine. In addition, characterizing the role of eIF2 α in the adaptive AA response and in response to other stressors could reveal greater insight into its function in milk synthesis. Ultimately, in vivo studies will be needed to establish the physiological relevance of the ISR pathway on milk protein production.

The CRISPR-Cas9 system has many applications in animal agriculture but has not been used extensively to study gene function. Recently, Tian et al. (2018) transfected goat mammary epithelial cells with CRISPR-Cas9 to demonstrate an important role for SCD1 in fatty acid synthesis. Here we demonstrate that this technology can also be deployed using lentiviral delivery to KO genes in primary BMEC. The implementation of this powerful genomic editing technology for characterization of gene function represents a major technical advance in the field that has the potential to deepen our understanding of the molecular mechanisms of milk synthesis.

CONCLUSIONS

We found that the combined deprivation of Arg, Leu, and Lys, sensed by GCN2, activates the ISR in BMEC. Notably, our results demonstrate that the individual absence of Arg in BMEC culture medium has a more pronounced effect on eIF2 α phosphorylation than the absence of Leu or Lys. In addition, we showed that both combined AA deprivation and the individual deprivation of Arg induced eIF2 α Ser51 phosphorylation for up to 8 h. We discovered that *ASNS* and *SLC7A1*, downstream gene targets of ATF4, specific to adaptation to AA deprivation, were upregulated in response to the combined deprivation of Arg, Leu, and Lys or Arg alone. By employing CRISPR-Cas9 to KO the ISR sensor GCN2, we confirmed its role in the adaptive response to AA deprivation in BMEC. Collectively, this

study demonstrates that GCN2 plays an important role in the response to AA deprivation and establishes the use of lentiviral-delivery CRISPR-Cas9 as a powerful tool to characterize gene function in primary BMEC.

ACKNOWLEDGMENTS

A. M. Edick received a Graduate Excellence Fellowship from McGill University. This work was funded by a new researcher's start-up grant from the Fonds de recherche du Québec–Nature et technologies (NC-205408) and a Discovery Grant from the Natural Sciences and Engineering Research Council (RGPIN-05007) to S. A. Burgos. The authors have not stated any conflicts of interest.

REFERENCES

- Appuhamy, J. A., A. L. Bell, W. A. Nayananjali, J. Escobar, and M. D. Hanigan. 2011. Essential amino acids regulate both initiation and elongation of mRNA translation independent of insulin in MAC-T cells and bovine mammary tissue slices. *J. Nutr.* 141:1209–1215. <https://doi.org/10.3945/jn.110.136143>.
- Appuhamy, J. A., N. A. Knoebel, W. A. Nayananjali, J. Escobar, and M. D. Hanigan. 2012. Isoleucine and leucine independently regulate mTOR signaling and protein synthesis in MAC-T cells and bovine mammary tissue slices. *J. Nutr.* 142:484–491. <https://doi.org/10.3945/jn.111.152595>.
- Aranda, P. S., D. M. LaJoie, and C. L. Jorcyk. 2012. Bleach gel: A simple agarose gel for analyzing RNA quality. *Electrophoresis* 33:366–369. <https://doi.org/10.1002/elps.201100335>.
- Arriola Apelo, S. I., L. M. Singer, X. Y. Lin, M. L. McGilliard, N. R. St-Pierre, and M. D. Hanigan. 2014. Isoleucine, leucine, methionine, and threonine effects on mammalian target of rapamycin signaling in mammary tissue. *J. Dairy Sci.* 97:1047–1056. <https://doi.org/10.3168/jds.2013-7348>.
- Auclair, O., Y. Han, and S. A. Burgos. 2019. Consumption of milk and alternatives and their contribution to nutrient intakes among Canadian adults: Evidence from the 2015 Canadian Community Health Survey-Nutrition. *Nutrients* 11:1948. <https://doi.org/10.3390/nu11081948>.
- Barrangou, R., C. Fremaux, H. Deveau, M. Richards, P. Boyaval, S. Moineau, D. A. Romero, and P. Horvath. 2007. CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 315:1709–1712. <https://doi.org/10.1126/science.1138140>.
- Basch, J. J., E. D. Wickham, and H. M. Farrell Jr.. 1997. Arginase in lactating bovine mammary glands: Implications in proline synthesis. *J. Dairy Sci.* 80:3241–3248. [https://doi.org/10.3168/jds.S0022-0302\(97\)76298-2](https://doi.org/10.3168/jds.S0022-0302(97)76298-2).
- Baumrucker, C. R. 1984. Cationic amino acid transport by bovine mammary tissue. *J. Dairy Sci.* 67:2500–2506. [https://doi.org/10.3168/jds.S0022-0302\(84\)81606-9](https://doi.org/10.3168/jds.S0022-0302(84)81606-9).
- Berlanger, J. J., J. Santoyo, and C. De Haro. 1999. Characterization of a mammalian homolog of the GCN2 eukaryotic initiation factor 2alpha kinase. *Eur. J. Biochem.* 265:754–762. <https://doi.org/10.1046/j.1432-1327.1999.00780.x>.
- Boratyn, G. M., A. A. Schaffer, R. Agarwala, S. F. Altschul, D. J. Lipman, and T. L. Madden. 2012. Domain enhanced lookup time accelerated BLAST. *Biol. Direct* 7:12. <https://doi.org/10.1186/1745-6150-7-12>.
- Burgos, S. A., M. Dai, and J. P. Cant. 2010. Nutrient availability and lactogenic hormones regulate mammary protein synthesis through the mammalian target of rapamycin signaling pathway. *J. Dairy Sci.* 93:153–161. <https://doi.org/10.3168/jds.2009-2444>.
- Clark, J. H. 1975. Lactational responses to postpartum administration of proteins and amino-acids. *J. Dairy Sci.* 58:1178–1197. [https://doi.org/10.3168/jds.S0022-0302\(75\)84696-0](https://doi.org/10.3168/jds.S0022-0302(75)84696-0).
- Clark, J. H., R. G. Derrig, C. L. Davis, and H. R. Spiers. 1975. Metabolism of arginine and ornithine in the cow and rabbit mammary tissue. *J. Dairy Sci.* 58:1808–1813. [https://doi.org/10.3168/jds.S0022-0302\(75\)84791-6](https://doi.org/10.3168/jds.S0022-0302(75)84791-6).
- Degasperis, A., M. R. Birtwistle, N. Volinsky, J. Rauch, W. Kolch, and B. N. Kholodenko. 2014. Evaluating strategies to normalise biological replicates of Western blot data. *PLoS One* 9:e87293. <https://doi.org/10.1371/journal.pone.0087293>.
- Doelman, J., J. J. Kim, M. Carson, J. A. Metcalf, and J. P. Cant. 2015. Branched-chain amino acid and lysine deficiencies exert different effects on mammary translational regulation. *J. Dairy Sci.* 98:7846–7855. <https://doi.org/10.3168/jds.2015-9819>.
- Doench, J. G., N. Fusi, M. Sullender, M. Hegde, E. W. Vaimberg, K. F. Donovan, I. Smith, Z. Tothova, C. Wilen, R. Orchard, H. W. Virgin, J. Listgarten, and D. E. Root. 2016. Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat. Biotechnol.* 34:184–191. <https://doi.org/10.1038/nbt.3437>.
- Doepel, L., and H. Lapierre. 2011. Deletion of arginine from an abomasal infusion of amino acids does not decrease milk protein yield in Holstein cows. *J. Dairy Sci.* 94:864–873. <https://doi.org/10.3168/jds.2010-3497>.
- Dong, X., Z. Zhou, B. Saremi, A. Helmbrecht, Z. Wang, and J. J. Loo. 2018. Varying the ratio of Lys:Met while maintaining the ratios of Thr:Phe, Lys:Thr, Lys:His, and Lys:Val alters mammary cellular metabolites, mammalian target of rapamycin signaling, and gene transcription. *J. Dairy Sci.* 101:1708–1718. <https://doi.org/10.3168/jds.2017-13351>.
- Fekete, Á. A., D. I. Givens, and J. A. Lovegrove. 2016. Can milk proteins be a useful tool in the management of cardiometabolic health? An updated review of human intervention trials. *Proc. Nutr. Soc.* 75:328–341. <https://doi.org/10.1017/S0029665116000264>.
- Gao, H. N., S. G. Zhao, N. Zheng, Y. D. Zhang, S. S. Wang, X. Q. Zhou, and J. Q. Wang. 2017. Combination of histidine, lysine, methionine, and leucine promotes beta-casein synthesis via the mechanistic target of rapamycin signaling pathway in bovine mammary epithelial cells. *J. Dairy Sci.* 100:7696–7709. <https://doi.org/10.3168/jds.2015-10729>.
- Garbe, J. C., S. Bhattacharya, B. Merchant, E. Bassett, K. Swisshelm, H. S. Feiler, A. J. Wyrobek, and M. R. Stampfer. 2009. Molecular distinctions between stasis and telomere attrition senescence barriers shown by long-term culture of normal human mammary epithelial cells. *Cancer Res.* 69:7557–7568. <https://doi.org/10.1158/0008-5472.CAN-09-0270>.
- Hara, K., K. Yonezawa, Q. P. Weng, M. T. Kozłowski, C. Belham, and J. Avruch. 1998. Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J. Biol. Chem.* 273:14484–14494. <https://doi.org/10.1074/jbc.273.23.14484>.
- Huang, J., M. A. Guesthler, and S. A. Burgos. 2020. AMP-activated protein kinase controls lipid and lactose synthesis in bovine mammary epithelial cells. *J. Dairy Sci.* 103:340–351. <https://doi.org/10.3168/jds.2019-16343>.
- Huhtanen, P., and A. N. Hristov. 2009. A meta-analysis of the effects of dietary protein concentration and degradability on milk protein yield and milk N efficiency in dairy cows. *J. Dairy Sci.* 92:3222–3232. <https://doi.org/10.3168/jds.2008-1352>.
- Jedrejczak, M., and I. Szatkowska. 2014. Bovine mammary epithelial cell cultures for the study of mammary gland functions. *In Vitro Cell. Dev. Biol. Anim.* 50:389–398. <https://doi.org/10.1007/s11626-013-9711-4>.
- Kilberg, M. S., J. Shan, and N. Su. 2009. ATF4-dependent transcription mediates signaling of amino acid limitation. *Trends Endocrinol. Metab.* 20:436–443. <https://doi.org/10.1016/j.tem.2009.05.008>.
- Labun, K., T. G. Montague, J. A. Gagnon, S. B. Thyme, and E. Valen. 2016. CHOPCHOP v2: A web tool for the next generation of

- CRISPR genome engineering. *Nucleic Acids Res.* 44(W1):W272–W276. <https://doi.org/10.1093/nar/gkw398>.
- Lapierre, H., G. E. Lobley, L. Doepel, G. Raggio, H. Rulquin, and S. Lemosquet. 2012. Triennial Lactation Symposium: Mammary metabolism of amino acids in dairy cows. *J. Anim. Sci.* 90:1708–1721. <https://doi.org/10.2527/jas.2011-4645>.
- Lin, X., S. Li, Y. Zou, F. Q. Zhao, J. Liu, and H. Liu. 2018. Lysine stimulates protein synthesis by promoting the expression of ATB0,+ and activating the mTOR pathway in bovine mammary epithelial cells. *J. Nutr.* 148:1426–1433. <https://doi.org/10.1093/jn/nxy140>.
- Mackenzie, B., and J. D. Erickson. 2004. Sodium-coupled neutral amino acid (System N/A) transporters of the SLC38 gene family. *Pflugers Arch.* 447:784–795. <https://doi.org/10.1007/s00424-003-1117-9>.
- Manjarin, R., B. J. Bequette, G. Wu, and N. L. Trottier. 2014. Linking our understanding of mammary gland metabolism to amino acid nutrition. *Amino Acids* 46:2447–2462. <https://doi.org/10.1007/s00726-014-1818-8>.
- Mephum, T. B. 1982. Amino acid utilization by lactating mammary gland. *J. Dairy Sci.* 65:287–298. [https://doi.org/10.3168/jds.S0022-0302\(82\)82191-7](https://doi.org/10.3168/jds.S0022-0302(82)82191-7).
- Moshel, Y., R. E. Rhoads, and I. Barash. 2006. Role of amino acids in translational mechanisms governing milk protein synthesis in murine and ruminant mammary epithelial cells. *J. Cell. Biochem.* 98:685–700. <https://doi.org/10.1002/jcb.20825>.
- Nan, X., D. Bu, X. Li, J. Wang, H. Wei, H. Hu, L. Zhou, and J. J. Loo. 2014. Ratio of lysine to methionine alters expression of genes involved in milk protein transcription and translation and mTOR phosphorylation in bovine mammary cells. *Physiol. Genomics* 46:268–275. <https://doi.org/10.1152/physiolgenomics.00119.2013>.
- Nichols, K., J. Doelman, J. J. M. Kim, M. Carson, J. A. Metcalf, and J. P. Cant. 2017. Exogenous essential amino acids stimulate an adaptive unfolded protein response in the mammary glands of lactating cows. *J. Dairy Sci.* 100:5909–5921. <https://doi.org/10.3168/jds.2016-12387>.
- NRC. 2001. *Nutrient Requirements of Dairy Cattle*. 7th rev. ed. ed. National Academy Press, Washington, DC.
- Pakos-Zebrucka, K., I. Koryga, K. Mnich, M. Ljubic, A. Samali, and A. M. Gorman. 2016. The integrated stress response. *EMBO Rep.* 17:1374–1395. <https://doi.org/10.15252/embr.201642195>.
- Pasiakos, S. M., S. Agarwal, H. R. Lieberman, and V. L. Fulgoni 3rd.. 2015. Sources and Amounts of Animal, Dairy, and Plant Protein Intake of US Adults in 2007–2010. *Nutrients* 7:7058–7069. <https://doi.org/10.3390/nu7085322>.
- Salama, A. A. K., M. Duque, L. Wang, K. Shahzad, M. Olivera, and J. J. Loo. 2019. Enhanced supply of methionine or arginine alters mechanistic target of rapamycin signaling proteins, messenger RNA, and microRNA abundance in heat-stressed bovine mammary epithelial cells in vitro. *J. Dairy Sci.* 102:2469–2480. <https://doi.org/10.3168/jds.2018-15219>.
- Sanjana, N. E., O. Shalem, and F. Zhang. 2014. Improved vectors and genome-wide libraries for CRISPR screening. *Nat. Methods* 11:783–784. <https://doi.org/10.1038/nmeth.3047>.
- Saxton, R. A., and D. M. Sabatini. 2017. mTOR signaling in growth, metabolism, and disease. *Cell* 168:960–976. <https://doi.org/10.1016/j.cell.2017.02.004>.
- Shalem, O., N. E. Sanjana, E. Hartenian, X. Shi, D. A. Scott, T. Mikkelsen, D. Heckl, B. L. Ebert, D. E. Root, J. G. Doench, and F. Zhang. 2014. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* 343:84–87. <https://doi.org/10.1126/science.1247005>.
- Taylor, S. C., K. Nadeau, M. Abbasi, C. Lachance, M. Nguyen, and J. Fenrich. 2019. The ultimate qPCR experiment: Producing publication quality, reproducible data the first time. *Trends Biotechnol.* 37:761–774. <https://doi.org/10.1016/j.tibtech.2018.12.002>.
- Tian, H., J. Luo, Z. Zhang, J. Wu, T. Zhang, S. Busato, L. Huang, N. Song, and M. Bionaz. 2018. CRISPR/Cas9-mediated stearoyl-CoA desaturase 1 (SCD1) deficiency affects fatty acid metabolism in goat mammary epithelial cells. *J. Agric. Food Chem.* 66:10041–10052. <https://doi.org/10.1021/acs.jafc.8b03545>.
- Vattem, K. M., and R. C. Wek. 2004. Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. *Proc. Natl. Acad. Sci. USA* 101:11269–11274. <https://doi.org/10.1073/pnas.0400541101>.
- Verrey, F., E. I. Closs, C. A. Wagner, M. Palacin, H. Endou, and Y. Kanai. 2004. CATs and HATs: The SLC7 family of amino acid transporters. *Pflugers Arch.* 447:532–542. <https://doi.org/10.1007/s00424-003-1086-z>.
- Wang, X., L. E. Campbell, C. M. Miller, and C. G. Proud. 1998. Amino acid availability regulates p70 S6 kinase and multiple translation factors. *Biochem. J.* 334:261–267. <https://doi.org/10.1042/bj3340261>.
- Weekes, T. L., P. H. Luimes, and J. P. Cant. 2006. Responses to amino acid imbalances and deficiencies in lactating dairy cows. *J. Dairy Sci.* 89:2177–2187. [https://doi.org/10.3168/jds.S0022-0302\(06\)72288-3](https://doi.org/10.3168/jds.S0022-0302(06)72288-3).
- Wolfson, R. L., and D. M. Sabatini. 2017. The dawn of the age of amino acid sensors for the mTORC1 pathway. *Cell Metab.* 26:301–309. <https://doi.org/10.1016/j.cmet.2017.07.001>.
- Wu, T., C. Wang, L. Ding, Y. Shen, H. Cui, M. Wang, and H. Wang. 2016. Arginine relieves the inflammatory response and enhances the casein expression in bovine mammary epithelial cells induced by lipopolysaccharide. *Mediators Inflamm.* 2016:9618795. <https://doi.org/10.1155/2016/9618795>.
- Xia, X., Y. Che, Y. Gao, S. Zhao, C. Ao, H. Yang, J. Liu, G. Liu, W. Han, Y. Wang, and L. Lei. 2016. Arginine supplementation recovered the IFN-gamma-mediated decrease in milk protein and fat synthesis by inhibiting the GCN2/eIF2alpha pathway, which induces autophagy in primary bovine mammary epithelial cells. *Mol. Cells* 39:410–417. <https://doi.org/10.14348/molcells.2016.2358>.
- Ye, J., G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen, and T. L. Madden. 2012. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 13:134. <https://doi.org/10.1186/1471-2105-13-134>.
- Ye, J., W. Palm, M. Peng, B. King, T. Lindsten, M. O. Li, C. Koumenis, and C. B. Thompson. 2015. GCN2 sustains mTORC1 suppression upon amino acid deprivation by inducing Sestrin2. *Genes Dev.* 29:2331–2336. <https://doi.org/10.1101/gad.269324.115>.
- Zhang, P., B. C. McGrath, J. Reinert, D. S. Olsen, L. Lei, S. Gill, S. A. Wek, K. M. Vattem, R. C. Wek, S. R. Kimball, L. S. Jefferson, and D. R. Cavener. 2002. The GCN2 eIF2alpha kinase is required for adaptation to amino acid deprivation in mice. *Mol. Cell. Biol.* 22:6681–6688. <https://doi.org/10.1128/MCB.22.19.6681-6688.2002>.

ORCIDS

A. M. Edick <https://orcid.org/0000-0003-2146-4798>
 S. A. Burgos <https://orcid.org/0000-0003-0569-4307>