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

Ashlin M. Edick

Department of Animal Science
McGill University, Montreal
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Abstract (English)

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 5-7 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 eIF2α 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 eIF2α Ser51 after 1 h of deprivation, respectively. In addition, activation of GCN2 as measured by increased phosphorylation of eIF2α Ser51 during the deprivation of Arg, Leu, and Lys combined and of Arg alone was sustained for up to 8 h of deprivation. Phosphorylated eIF2α 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 deprivation 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 RNAs targeting *EIF2AK4* were not as responsive to combined AA deprivation, compared to BMEC transduced with non-targeting single-guide RNAs. Taken together, our results demonstrate a critical role for GCN2 in the adaptive response of BMEC to AA deprivation.

Abstract (French)

Le but de cette étude était de vérifier le rôle que joue GCN2 lorsque les cellules épithéliales mammaires bovines (BME) de culture primaire étaient dépourvues d'acides aminés (AA). Les cellules ont été isolées du tissu mammaire de deux vaches allaitantes Holstein par digestion enzymatique, multipliée et différenciée pour 5 à 7 jours. L'expression relative de l'ARNm a été mesurée par l'ACP quantitatif en temps réel. L'abondance de protéines et la phosphorylation site-spécifique ont été mesurées par western blot. L'invalidation du GCN2 en BME a été accomplie par livraison d'un vecteur lentiviral d'un seul ARN guide ciblé et l'endonucléase Cas9. Pour examiner le rôle de GCN2, nous avons traité BME différencié en réponse d'hormones lactogéniques avec un milieu de culture dépourvu d'Arg, Leu et Lys combiner ou dépourvu d'un des trois AA d'intérêts comparer à un contrôle avec un complément entier d'AA. L'activation de GCN2 a été inférée par l'état de phosphorylation de sa cible située en aval, eIF2α Ser51. Nous avons trouvé que GCN2 a été activé lorsque BME étaient dépourvues d'Arg, Leu et Lys combinées et d'Arg seul, comme démontré par un accroissement de 2.73 et 2.82 de eIF2α Ser51 phosphorylé à 1 heure de traitement, respectivement. De plus, l'activation de GCN2, mesurée par un accroissement de phosphorylation de eIF2α Ser51 par l'absence d'Arg, Leu et Lys combinées et d'Arg seule, a été soutenue jusqu'à 8 heures de traitement. L'eIF2α phosphorylé régule positivement la traduction du facteur de transcription ATF4, parmi d'autres, par l'absence d'AA qui ciblent les gènes nécessaires pour restituer l'homéostasie d'AA. Par conséquent, nous avons examiné l'expression des cibles transcriptionnelles d'ATF4, l'enzyme d'AA *ASNS* et les transporteurs d'AA *SLC7A1* et *SLC38A2*. Nous avons trouvé que *ASNS* a été régulé positivement lorsque BME étaient dépourvues d'AA combiner et d'Arg seule, démontrer par un accroissement de 3.6 et 4.51, respectivement, à 24 heures de traitement. Nous

avons aussi trouvé que *SLC7A1* a été régulé positivement par l'absence d'AA combiner et d'Arg seul, démontrer par un accroissement de 2.0 et 2.36, respectivement, à 8 heures de traitement. Pour établir le rôle de GCN2 (encoder par *EIF2AK4*) lorsque BME étaient dépourvues d'AA, nous avons ablati GCN2 dans BME en utilisant les courtes répétitions palindromiques groupées et régulièrement espacées/Cas9. Nous avons démontré que BME transduit avec un seul ARN guide ciblant by *EIF2AK4* n'a pas été si responsif par l'absence d'AA comparé à BME transduit avec un seul ARN guide non ciblant. En définitive, nos résultats démontrent un rôle critique pour GCN2 à propos de la réponse adaptative de BME lorsqu'elles étaient dépourvues d'AA.

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Contribution of Authors

The authors involved in this thesis and their contributions to the various articles are as follows:

Ashlin EDICK is the M.Sc. Candidate who designed and performed all experiments in consultation with her principal supervisor and committee. She collected and analysed data. She prepared drafts of manuscripts and figures for scientific publication.

Dr. Sergio BURGOS is the thesis supervisor under whose guidance the research was conducted. He assisted the candidate in designing and performing the experiments as well as proofreading, reviewing, and processing the manuscripts for publication.

Julianne AUDETTE assisted in performing select experiments under the supervision of Ashlin EDICK and Dr. Sergio BURGOS.

The literature review was drafted and revised by Ashlin EDICK under the supervision of Dr. Sergio BURGOS. Introduction, Discussion, and Conclusions were drafted and revised by Ashlin EDICK.

Chapter 1 – Introduction

Nutritional Benefits and Environmental Impact of Dairy Industry

Nutritional and Health Benefits of Dairy Protein

Dairy products are a source of high-quality protein as well as essential vitamins and minerals for humans (Drewnowski and Fulgoni, 2008), contributing 16% of protein intake by adults in Canada and the United States (Pasiakos et al., 2015, Auclair et al., 2019). In addition, intake of dairy protein promotes maintenance of skeletal muscle mass and may contribute to beneficial cardiometabolic health effects (Bonjour et al., 2013, Fekete et al., 2016). A recent meta-analysis concluded that the consumption of high-quality protein from dairy products is an important nutritional intervention strategy to promote muscle health (Hanach et al., 2019). A systematic review by Drouin-Chartier et al. (2016) also found an inverse association between total dairy consumption and cardiovascular disease in several studies, whereas another study did not find an association. Evidence from this review also demonstrated that there was no association between dairy product consumption and coronary artery disease. Furthermore, consuming dairy products was associated with decreased risk of stroke and hypertension (Drouin-Chartier et al., 2016). Specifically, milk consumption was associated with a lowered risk of hypertension. Thus, milk not only serves a high-quality source of protein for humans (Hoffman and Falvo, 2004, Wolfe, 2015), but its intake is also positively associated with musculoskeletal and cardiometabolic health outcomes.

Nitrogen pollution from dairy production

Despite the nutritional and health benefits of dairy protein consumption, milk production contributes to environmental pollution. Ammonia emitted from animal feeding operations is a major water and air pollutant (Hristov, 2011). Excess nitrogen (N) is excreted in urine and feces

and then lost to the environment by leaching, run-off, and volatilization, where it can pose detrimental impacts through ecosystem fertilization, acidification, and eutrophication. Ammonia can also react with atmospheric acids to form fine particulate matter, becoming an air pollutant, which can be detrimental to human health even in low concentrations (Oberdorster et al., 2000, Miller et al., 2007). Thus, reducing ammonia emissions from dairy cattle farming is an important part of improving the environmental sustainability of the dairy industry.

N pollution from the dairy industry is largely due to the inefficiency of dairy cows in converting dietary N from protein and non-protein sources into milk protein (Huhtanen and Hristov, 2009). Transfer efficiency of feed N into milk protein is estimated to be between 25% and 28% (Bequette et al., 2003, Huhtanen and Hristov, 2009). The dietary N that is not captured by the mammary gland for synthesis of milk protein is eventually lost mainly as urine and feces. The three main factors contributing to ammonia emissions from cattle manure are the inefficient utilization of feed N in the rumen, inaccurate prediction of the animal degradable and undegradable protein requirements (leading to the overfeeding of dietary N), and underestimation of urea recycling to the rumen as a mechanism of N preservation (Hristov, 2011). Improving predictions of protein requirements is one of the most important ways to reduce N loss from dairying by preventing overfeeding of dietary protein (Kalscheur et al 2006; Huhtanen and Hristov, 2009). This is because current strategies advocate for the excess feeding of dietary protein to ensure desired milk protein yield, as it is increased by metabolizable protein supply (i.e., the protein reaching the small intestine so that it can be absorbed as amino acids) (Daniel et al., 2016). Milk protein secretion represents half of the metabolizable protein supply, implicating the mammary gland as the major net consumer of circulating amino acids (Lapierre et al., 2012). Milk protein production accounts for 90% of amino acid uptake by the mammary

gland (Cant et al., 1993). Therefore, understanding the mechanisms regulating amino acid utilization for milk protein synthesis may provide the key to increasing the amount of N incorporated into milk proteins and therefore reduce the amount of N released into the environment.

Chapter 2 – Literature Review

General Aspects of Amino Acids Nutrition

In addition to serving as the building blocks for proteins, amino acids (AA) play multiple roles in regulation of growth, signalling, nutrient metabolism, and oxidative defense (Yao et al., 2008, Brasse-Lagnel et al., 2009, Bruhat et al., 2009). AA that cannot be synthesized by the animal *de novo* or in adequate quantities are known as essential AA (EAA). In turn, AA that can be synthesized *de novo* in the cell are classified as non-essential AA (NEAA) (Wu, 2009), although they too are important for physiological function (Reeds, 2000). Of the twenty proteogenic AA, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, and Arg are essential for lactating dairy cows according to the (NRC, 2001). Furthermore, AA can be nutritionally classified as *limiting* to describe those that are in the shortest supply in the diet in relation to the maintenance, growth, and health requirements of the animal (Wu et al., 2013). This is known as the limiting AA theory. The current view is that milk protein production is limited by the AA that is present in the lowest quantity (i.e., the limiting AA), which are embodied in current dietary protein requirements for dairy cattle. However, the optimal levels of individual AA to sustain milk protein production in lactating dairy cattle have not been identified and therefore, cannot be specified in nutritional models for lactating dairy cows (NRC, 2001, Van Amburgh et al., 2015). Though, studies have shown that certain AA and AA ratios have a greater impact on milk protein production than others (Lapierre et al., 2012, Manjarin et al., 2014). For this reason, this literature review will primarily focus on Met, Lys, Arg, and Leu with regards to the effects of these AA on milk protein production in cows and the molecular mechanisms by which they may regulate protein synthesis in mammary epithelial cells.

Rumen N metabolism

Ruminants have evolved a unique system of N digestion that involves microbial fermentation of feed protein before digestion by the animal's own enzymes (McDonald et al., 2011). Rumen microorganisms hydrolyze feed proteins into peptides and AA, with some AA being degraded further into organic acids, ammonia, and carbon dioxide (McDonald et al., 2011). Ammonia, free AA, and small peptides obtained by breakdown of the protein and non-protein N fraction of feed are used to synthesize microbial protein. Rumen microorganisms are eventually carried through the omasum and abomasum into the small intestine where their cellular proteins are digested and the resulting AA are absorbed. In diets commonly fed to dairy cows, most of the protein that reaches the intestine is of microbial origin, with the minority being composed of rumen undegradable protein from feed (McDonald et al., 2011). Rumen undegradable protein will vary depending on the rumen degradability of the protein source. Ammonia is a key part of this process, as it is an intermediate in microbial degradation and synthesis. If ammonia concentration in rumen is low, it delays microorganism growth and carbohydrate breakdown. However, if ammonia concentration is high, it is absorbed through the rumen wall into the blood and carried to the liver where it is converted into urea. Urea is largely excreted in the urine and potentially contributes to N pollution (Lapierre and Lobley, 2001). This complex rumen N metabolism determines the amount of protein that eventually reaches the small intestine for breakdown and absorption.

Milk protein production and AA catabolism largely determine dairy cattle requirements for metabolizable protein, the true protein that is digested post-ruminally, and the component AA absorbed in the small intestine (Lapierre et al., 2012). Microbial crude protein synthesized in the rumen, undegraded feed crude protein, and to a lesser extent endogenous crude

protein, contribute to the passage of metabolizable protein to the small intestine (NRC, 2001). It has been suggested that an ideal profile of AA can improve efficiency of metabolizable protein utilization (Vyas and Erdman, 2009; Ding et al 2019). Improved metabolizable protein efficiency would be beneficial for dairy farmers by decreasing cost of expensive feed components while also reducing the environmental impact of dairying.

Effect of dietary protein and amino acids on milk protein production

Feed trials testing the effects of dietary protein sources on milk protein production in dairy cows have yielded inconsistent results. This could be due in part to the various types of dietary protein that are degraded at different rates thereby affecting the profile of AA that are made available when they are metabolized by dairy cattle (Baker et al., 1995). Indeed, early studies suggested that for cows fed common corn silage- and alfalfa-based diets, Lys and Met are the most limiting AA (Schwab et al., 1976). However, Lys or Met supplementation for a 14 d period did not improve milk protein yield when cows are fed grass silage-based diets (Varvikko et al., 1999). In cattle fed grass silage-based diets, only His supplementation appeared to improve milk protein yield over a 14 d period (Vanhatalo et al., 1999). Differences in AA composition and rumen degradability among dietary protein sources has led to difficulty in determining which AA are key to maximizing milk protein production based on feed trials.

Feed trial testing post-ruminal supplementation of Lys and Met with rumen-protected AA have also led to variable results. For instance, a study in early lactation Holstein cows showed that supplementation with rumen-protected Met maximized milk protein yield and N efficiency (Noftsger and St-Pierre, 2003). Subsequent studies with cows ranging from approximately 50 to 130 d in milk demonstrated that supplementing lactating Holstein cows fed diets deficient in metabolizable protein with rumen-protected Lys and Met both increased milk protein yield (Lee

et al., 2012, Zhao et al., 2019). Another study using multiparous Holstein cows in mid-lactation fed a basal total mixed ration containing ample Lys did not observe any effects of rumen-protected Met on milk protein yield (Benefield et al., 2009). Socha et al. (2005) showed that rumen-protected Lys and Met, but surprisingly not Met alone, increased milk protein content when fed to cows both pre- and post-partum. Importantly, the authors noted that the milk protein response of early lactation cows to Lys and Met supplementation was dependent on crude protein concentration, metabolizable protein supply, and intestinal digestibility of the rumen undegradable proteins (Socha et al., 2005). A meta-analysis of feed trials in lactating dairy cows that supplemented rumen-protected Lys and Met combined showed that they can increase milk protein yield as well as efficiency of converting dietary protein to milk protein (Vyas and Erdman, 2009).

Leucine has also been proposed to be a limiting AA during early lactation (Larsen et al., 2014), but evidence for increased milk protein yield in response to supplementation is lacking. Supplementation of mid-lactation cows with rumen-protected Leu did not affect milk protein yield (Krizova et al., 2008). Similarly, feeding rumen-protected branched chain AA (BCAA) – Leu, Ile, and Val – did not affect milk component synthesis in early lactation cows that were fed a protein-restricted diet (Leal Yepes et al., 2019). Taken together, feed trials on rumen-protected AA have led to results that are inconsistent with the limiting amino acid theory, highlighting our incomplete understanding of post-ruminal AA metabolism and mechanisms that control milk protein production in dairy cows.

Effect of post-ruminal protein and amino acid infusions on milk protein production

Due to the extensive ruminal metabolism of dietary protein and variable flow of rumen-protected AA from the rumen, studies have used various methods to infuse highly digestible

proteins or AA solutions post-ruminally. These methods are used to test the effect AA supply on milk protein production. In the abomasal infusion technique, catheters are surgically installed into the abomasum or a tube is fitted through a rumen fistula to deliver known quantities of purified protein or AA infusates. Early studies demonstrated that abomasal infusion of casein increased both milk and milk protein yield (Broderick et al., 1970, Derrig et al., 1974, Vik-Mo et al., 1974, Schwab et al., 1976). Likewise, duodenal infusion of casein where cows are surgically fitted with duodenal cannulas, has shown that infusion increases milk protein yield (Guinard and Rulquin, 1994). However, there was a lack of consistency seen in post-ruminal protein infusion studies (Schwab et al., 1976), possibly due to a mismatch between the needs of the animal and the AA composition of the test protein on one or more specific AA.

Unlike feed trials with dietary proteins and supplemental rumen-protected AA, post-ruminal infusion of AA can give a clearer picture of the relationship between intestinal AA supply and milk protein production. Indeed, post-ruminal infusion of individual AA established that Lys and Met were limiting for milk protein production (Schwab et al., 1976). It was also shown that improved Lys and Met provision decreased the amount of dietary crude protein needed in order to achieve similar milk protein yields (Robert et al., 1989, Rulquin et al., 1990). Experiments done on six multi-catheterized dairy cows with abomasal infusions of AA or with altered Lys concentrations demonstrated that the decrease of metabolizable Lys by more than one third of recommended amounts caused a decrease in true milk protein (Lapierre et al., 2009). Intestinal supply of Lys and Met together or Met alone to cows during early lactation increased milk protein yield (Socha et al., 2005).

BCAA may also affect milk protein synthesis, but the consequences of deletion and addition studies where one or more AA are removed or added from the post-ruminal infusate

solution have varied. Unlike Lys, deletion of BCAA from an AA abomasal infusion did not affect milk protein yield (Weekes et al., 2006). However, duodenal infusion studies show that a decrease in the amount of either metabolizable Leu or Val decreased milk protein yield (Rulquin and Pisulewski, 2006, Haque et al., 2013). Subtraction of Ile from a duodenal infusion did not affect milk protein yield (Haque et al., 2013). Abomasal infusion of BCAA did not affect milk protein yield when cows were fed diets with ample metabolizable protein (Mackle et al., 1999). Still, abomasal infusion of a solution deficient in all three BCAA decreased milk protein yield (Doelman et al., 2015). In addition, milk protein yield increases were observed in response to the duodenal infusion of Leu alone (Rulquin and Pisulewski, 2006). Overall, removal of BCAA from AA infusates caused greater milk protein responses than in supplementation studies in lactating dairy cows, but the variability within and across studies indicate that further research is needed to clarify the effects of BCAA on milk protein yield.

Arginine is another AA that may affect milk protein synthesis, as it is important in meeting requirements of NEAA, primarily proline, found in milk protein. However, deletion of Arg from abomasal infusion, when all other AA were present, did not have an effect on milk protein synthesis (Doepel and Lapierre, 2011). Though, the addition of Arg to an abomasal infusion increased milk protein yield (Doepel and Lapierre, 2011). A duodenal infusion lacking Arg did not show a significant effect on milk protein or efficiency of N utilization (Haque et al., 2013). A consensus on the *in vivo* effects of either the addition or the deletion of Arg cannot be established due to the variation in both experimental design and outcome.

Intravenous infusion studies of AA have also been used to assess the effects of specific AA on milk protein yield (Fisher, 1972, Metcalf et al., 1996, Kim et al., 1999). In early-lactation Holstein cows fed a low protein diet, with infusions of Lys and Met increased, whereas deletion

of Lys decreased, milk protein yield (Weekes et al., 2006). Additionally, 7 d continuous jugular infusion of Lys and Met in early-lactation Holstein cows had positive effects on milk protein synthesis (Appuhamy et al., 2011). Furthermore, Yoder et al. (2020) found that jugular infusion of Met, Lys and His increased milk protein yield in cows approximately 99 d in milk. In this same series of experiments, they also found that infusion of Ile and Leu, two of the 3 BCAA, increased milk protein yield (Yoder et al., 2020). Milk protein yield was also increased when all 5 AA (Met, Lys, His, Ile and Leu) were combined (Yoder et al., 2020). Whereas, previously jugular infused BCAA in addition to Lys and Met did not show any significant effect in milk protein yield (Appuhamy et al., 2011). During 5 d continuous jugular EAA infusions with specific AA deletions into mid-lactation Holsteins, the absence of either Leu or Arg decreased milk protein yield (Tian et al., 2017). This is at odds with previous findings that intravenous injection of large amounts of Arg did not appear to change milk protein yields (Vicini et al., 1988). However, a recent jugular-infusion supplementation of Arg study demonstrated positive effects on milk protein yield in addition to improved N efficiency (Ding et al., 2019). Collectively, jugular infusion studies show that individual or groups of AA can elicit different effects on milk protein synthesis, however, consistent effects of individual AA remain to be seen.

Amino Acids Sensing Pathways

Regulation of Milk Protein Synthesis in Mammary Epithelial Cells

Bovine mammary epithelial cells (**BMEC**) are organized into alveoli (i.e., acini), the functional unit of mammary gland's secretory tissues (Park et al., 1979). Mammary epithelial cells uptake blood-borne AA to synthesize mammary-specific milk proteins, including casein and whey. Milk protein synthesis is responsible for greater than 90% of AA uptake by the mammary gland (Cant et al., 1993). Thus, understanding the mechanisms that govern mRNA

translation (i.e., protein synthesis) in BMEC in response to AA availability is an important pre-requisite to determining how to optimize milk protein synthesis. Specifically, a better understanding of how mammary cells sense AA levels and how signaling cascades act to restore the AA homeostasis when fluctuations in AA supply occur could lead to the design of feeding strategies that negate negative environment impacts on milk protein production. This thesis focuses on the two key signal transduction pathways by which mammalian cells sense and adapt to fluctuations in the intracellular AA concentrations: the integrated stress response (**ISR**) and mechanistic target of rapamycin complex 1 (**mTORC1**).

The ISR Pathway

The ISR is activated in response to a diverse array of stress stimuli including extrinsic factors such as viral infection, hypoxia, glucose deprivation, and AA deprivation, as well as intrinsic factors such as endoplasmic reticulum stress and oncogene activation (Pakos-Zebrucka et al., 2016). The eukaryotic initiation factor (eIF) 2 is a trimeric complex composed of α, β, and γ subunits that controls a rate-limiting step in the initiation of mRNA translation (Kimball, 1999). There are four ISR kinases that phosphorylate eIF2α at Ser51: double-stranded RNA-dependent protein kinase (PKR), PKR-like ER kinase (PERK), heme-regulated eIF2α kinase (HRI) and general control non-derepressible 2 (GCN2). Each of the eIF2α kinases dimerizes and autophosphorylates when activated by its unique environmental or physiological stressor (Pakos-Zebrucka et al., 2016). Normally, eIF2β activates the eIF2 complex by exchanging GDP for GTP thus converting eIF2 to its active form. The eIF2 complex can then form a ternary complex with GTP and Met-tRNA$_i$ that binds to the 40S ribosome subunit, an important step in forming the pre-initiation complex and eIF4F complex facilitated cap-dependent initiation of global protein synthesis (Aitken and Lorsch, 2012, Lomakin and Steitz, 2013). Phosphorylation of

eIF2α by any one of these stress sensors results in the global attenuation of cap-dependent translation through inhibition guanine nucleotide exchange factor eIF2β. In tandem with downregulation of global protein synthesis, the translation of ISR-specific mRNAs, including activating transcription factor 4 (ATF4) which is the main effector of the ISR, is initiated. Selected mRNAs that include a short-upstream open reading frame (ORF) in their 5' untranslated region, that are not reliant on cap recognition by the eIF4F complex, instead relying on a re-initiation mechanism or direct recruitment of ribosomes to internal ribosomal entry sites, are preferentially translated (Hinnebusch, 2011, Chan et al., 2013). *ATF4* in particular has two upstream ORF located in the 5' untranslated region (**Figure 2.1**). The two ORF are both translated during normal conditions, which prevents the production of full-length ATF4 protein, as the second ORF is out of frame with the ATF4 coding sequence (Vattem and Wek, 2004). However, under stress conditions, due to limited ternary complex availability leading to longer ribosomal scanning, only the first ORF is initiated and ATF4 is translated (Kilberg et al., 2009, Pakos-Zebrucka et al., 2016). ATF4 then binds to DNA targets to increase expression of genes involved in cellular adaptation, including AA transporters and biosynthetic AA enzymes (Kilberg et al., 2009). The increase in the translation of these specific proteins helps reverse AA deprivation initially sensed by GCN2 and re-establish AA homeostasis (Kilberg et al., 2009).

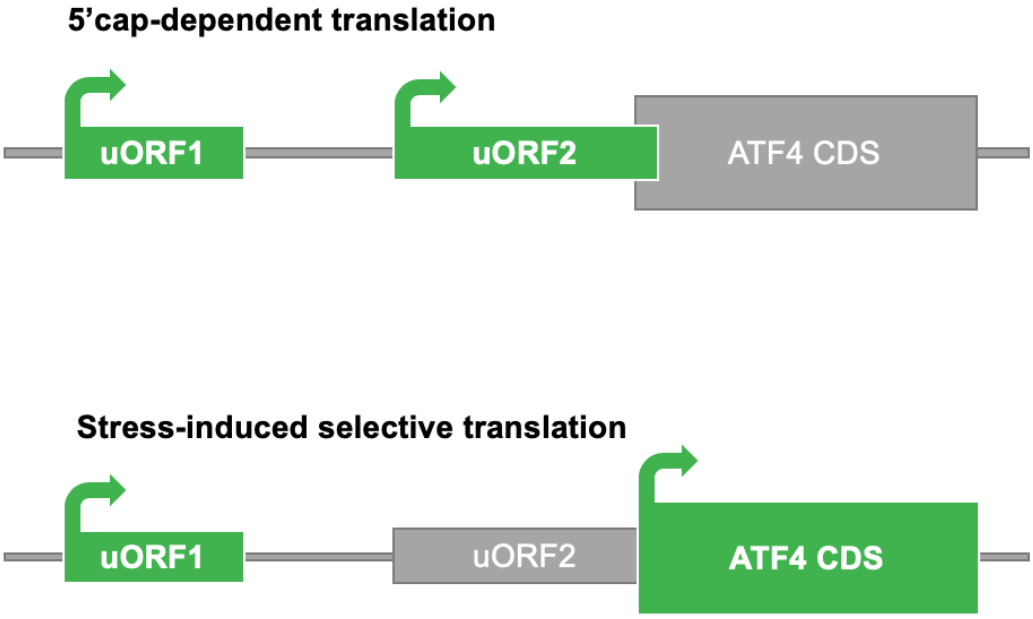

Figure 2.1. Model for translational control of ATF4. Scanning of ATF4 transcript during normal conditions: ATF4 mRNA is initiated at uORF1 and then re-initiated at uORF2. uORF2 sequence overlaps with ATF4 CDS in an out of frame manner precluding translation of ATF4 mRNA. Scanning of ATF4 transcript during amino acid deprivation conditions: ATF4 mRNA is initiated at uORF1, scans through uORF2, and is re-initiated at ATF4 CDS enabling ATF4 translation.

Amino acid Deprivation Sensor GCN2

GCN2 is a highly conserved eIF2α kinase that regulates the adaptive action of the ISR pathway in response to AA levels (**Figure 2.2**). During conditions of single AA deprivation, the intracellular accumulation of uncharged (unacylated) tRNA activates GCN2 leading to phosphorylation of eIF2α at Ser51, a critical control point in mRNA translation, which results in general inhibition of protein synthesis (Berlanga et al., 1999). By suppressing protein synthesis, eIF2α reduces the need for AA and staves off further AA depletion. Phosphorylation

of eIF2α by GCN2 also promotes the selective translation of ATF4, a transcription factor that induces the expression of genes that enable adaptation to AA scarcity in order to restore AA homeostasis (Vattem and Wek, 2004). The physiological role of GCN2 in response to AA deprivation has been studied using transgenic mice. GCN2 knockout (KO) mice were viable, fertile and had no phenotypic abnormalities under standard growth conditions (Zhang et al., 2002). However, they were unable to develop normally during conditions of AA deprivation (Zhang et al., 2002). This highlights the adaptive nature of the pathway activity in that GCN2 ablation is only noticeable when it is activated by AA scarcity to restore homeostasis through activation of downstream targets.

ATF4 and downstream targets

The transcription factor ATF4 binds to CCAAT-enhancer binding protein-activation transcription factor (C/EBP-ATF) Response Elements (CARE) in the promoter regions of genes involved in adaptation to AA stress triggering increased transcription (Fawcett et al., 1999). ATF4 regulates numerous genes involved in AA transport, metabolism, oxidation status, and energy management (Figure 2.2). In the adaptive response to AA deprivation, ATF4 targets genes involved in AA transport, including solute carrier family 7 member 1 (*SLC7A1*) and solute carrier family 38 member 2 (*SLC38A2*), encoding transporters cationic amino acid transporter 1 (CAT1) and sodium coupled neutral amino acid transporter 2 (SNAT2), respectively, as well as biosynthetic enzyme asparagine synthetase (*ASNS*) which is involved in asparagine and glutamate synthesis (Kilberg et al., 2009). This is important in restoring AA homeostasis which is dependent upon transport and exchange of essential AA with non-essential AA and the transfer of amino groups from oxidized AA to AA biosynthesis (Broer and Broer, 2017). Other targets are also preferentially translated during AA deprivation including negative regulator growth

arrest and DNA damage inducible protein 34 (GADD34), encoded by protein phosphatase 1 regulatory subunit 15A (*PPP1R15A*) which when mediated by protein phosphatase 1 (PP1), dephosphorylates eIF2α (Brush et al., 2003). C/EBP homologous protein (*CHOP*), *C/EBPβ* and activating transcription factor 3 (*ATF3*) are also upregulated by ATF4 and are able to limit ATF4 activity, however, the exact mechanism by which they accomplish this is unknown (Kilberg et al., 2009). *CHOP* can also act with ATF4 to inhibit *ASNS* synthesis and induce the unfolded protein response (UPR) pathway (Su and Kilberg, 2008). Recent studies summarized by Pakos-Zebrucka et al. (2016) suggest that CHOP also plays an important role in autophagy during nutrient starvation. In addition, ATF4 is required for autophagy induction, however, it has been suggested that other mechanisms are also involved (Kroemer et al., 2010, Pakos-Zebrucka et al., 2016). In addition to autophagy, CHOP can induce apoptosis through upregulation of pro-apoptotic bcl-2-like protein 11 (BCL2L11), bcl-2-binding component 3 (BBC3) and death receptor 5 (DR5) as can be seen in Figure 2.2 (Pakos-Zebrucka et al., 2016).

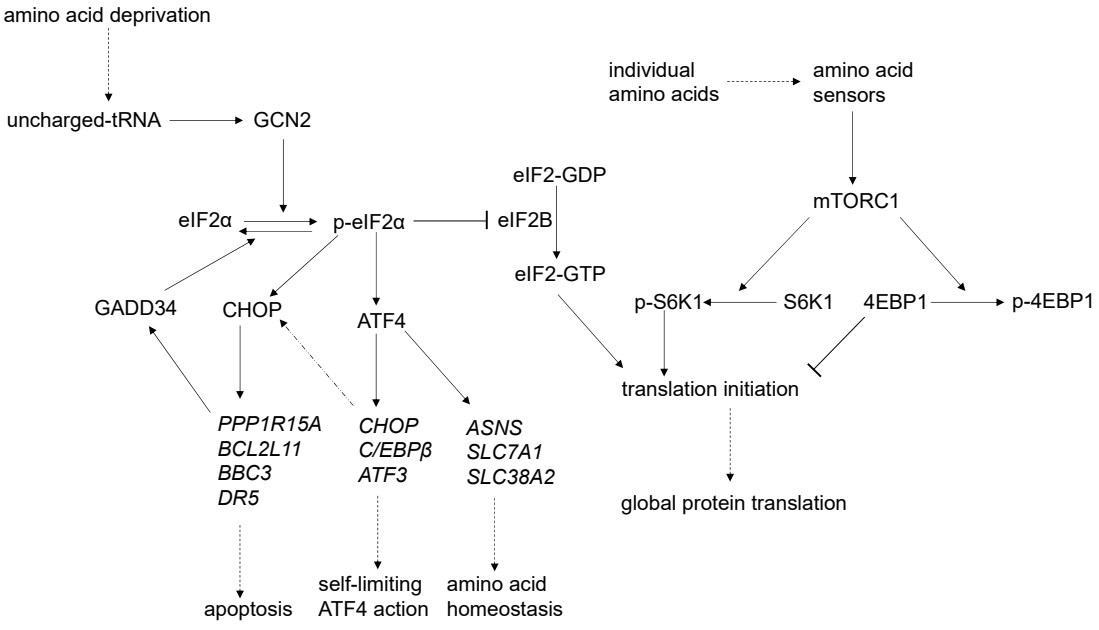

Figure 2.2. Regulation of translation initiation by the ISR and mTORC1 signalling pathways in response to amino acid levels. On the far left, the response of ISR pathway to AA deprivation sensed by GCN2 and the subsequent attenuation of translation initiation in tandem with preferential translation of adaptive gene targets of ATF4. On the right, mTORC1 regulates translation initiation through downstream targets S6K1 and 4EBP1 in response to individual amino acid levels. Solid arrows represent activation, blunt-ended arrows represent inactivation, and dashed arrows represent pathway environmental stimulus and effects.

The mTORC1 pathway

Mechanistic target of rapamycin complex 1 (mTORC1) acts as a regulator of metabolism, translation, and autophagy (Condon and Sabatini, 2019). mTORC1 is responsive to a variety of environmental signals including growth factors, cellular stresses, energy, and AA levels (Saxton and Sabatini, 2017). When present, specific AA are able to stimulate mTORC1 activity and upregulate protein synthesis primarily through mTORC1 downstream targets including ribosomal protein S6 kinase beta-1 (S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), both of which play a role in control of translation initiation (Figure 2.2). Once mTORC1 has phosphorylated S6K1 at Thr389, phosphorylated S6K1 can be activated by phosphoinositide-dependent kinase 1 (PDK1), which then activates various substrates involved in mRNA translation initiation including eIF4B. Phosphorylated eIF4B is recruited to the preinitiation complex and is an important positive regulator of the 5' binding eIF4F complex (Holz et al., 2005). Unphosphorylated 4EBP1 inhibits translation by binding eIF4E and therefore preventing assembly of the eIF4F complex; mTORC1 phosphorylation of 4EBP1 prevents this inhibitory activity allowing translation initiation to proceed (Gingras et al., 1999). Amino acids influence mTORC1 regulated protein synthesis through upstream sensors

that bind to AA or AA derived molecules at the point of Rag GTPases (Sancak et al., 2008). For example, Leu and Arg convey information about AA sufficiency to mTORC1 through binding to Sestrin2 and Castor1, respectively, which both interact with Gator2 (Saxton and Sabatini, 2017, Wolfson and Sabatini, 2017).

Amino acids are important signaling molecules for the translational machinery responsible for protein synthesis. Recent advances in identifying and characterizing upstream AA sensors have shown that Leu and Arg both have the capability of signaling Rag GTPase-mediated upregulation of mTORC1 driven translation initiation (Chantranupong et al., 2016, Wolfson et al., 2016). Sestrins, specifically Sestrin2, were the first identified AA sensors that regulate the mTORC1 pathway in response to BCAA (Budanov and Karin, 2008, Saxton et al., 2016b, Wolfson et al., 2016). Subsequently, Castor1 was identified as a cytosolic sensor of Arg sensor upstream of mTORC1 (Chantranupong et al., 2016, Saxton et al., 2016a). Sestrin2 and Castor1 are both capable of binding to Gator2, preventing it from binding and inactivating Gator1, a negative regulator of Rag GTPases (Bar-Peled et al., 2013). Sestrin2 and Castor1 bind to their respective AA, Leu and Arg, when the AA are present in sufficient concentrations and dissociate from Gator2 (Chantranupong et al., 2014, Chantranupong et al., 2016); unbound Gator2 is available to bind to negative regulator Gator1 preventing it from inhibiting Rag GTPases (Bar-Peled et al., 2013, Parmigiani et al., 2014). Rag GTPases consist of heterodimers of either RagA or RagB bound to RagC or RagD with their GTP loading state dictated by nutrient availability (Sekiguchi et al., 2001, Sancak et al., 2008). Rag GTPases are important for AA-induced mTORC1 recruitment to the lysosome, where the complex can then be completely activated by kinase activator Rheb (Saito et al., 2005, Buerger et al., 2006, Sancak et al., 2008).

Once mTORC1 is activated, it promotes global translation through phosphorylation of target proteins involved in protein synthesis.

Crosstalk between ISR and mTORC1 Pathways

Activation of the ISR by GCN2 leads to the attenuation of global protein synthesis, which is regulated by mTORC1 under normal conditions. Suppression of the mTORC1 pathway, which normally enables protein synthesis by phosphorylation-induced changes of multiple components of the translational machinery activity including S6K1 and 4E-BP1 (Hara et al., 1998, Wang et al., 1998), is accomplished through upregulation of ATF4 target, REDD1 (Ye et al., 2015, Pakos-Zebrucka et al., 2016). In addition, in response to AA deprivation, GCN2 activation and subsequent ATF4 increase transcriptionally upregulates Sestrin2, a negative upstream regulator of mTORC1 (Ye et al., 2015). When Leu levels are low, Sestrin2 blocks lysosomal recruitment and activation of mTORC1, an important regulator of protein metabolism (Condon and Sabatini, 2019). The crosstalk between these two pathways shows that there is a multi-factorial response to AA deprivation involving changes in both signalling pathways.

Effects of Arg, Leu, and Lys in vitro

Methionine, Lys, Arg and the BCAA, specifically Leu, are taken up in quantities that exceed those needed for milk protein production (Manjarin et al., 2014). Methionine and Lys are often the first or second limiting AA in cows with the majority designated for milk protein synthesis in mammary tissues (Manjarin et al., 2014). Uptake of Pro by the mammary gland is not sufficient to support casein production (Clark et al., 1975). However, proline can be synthesized from uptake of precursor AA in excess including Arg and ornithine (Mepham, 1982). Proline synthesis proceeds in a dose-dependent manner with the hydrolysis of Arg to urea

and ornithine by arginase present in the bovine mammary gland (Basch et al., 1997). Thus, Arg supply is important for NEAA proline to be synthesized in quantities needed for casein synthesis and for maintenance of AA homeostasis (Clark et al., 1975, Mepham, 1982). Similarly, some of the excess BCAA taken up by the mammary gland are incorporated into aspartate and glutamate, which are both taken up in lower quantities than is output into milk protein (Clark, 1975, Wohlt et al., 1977). Thus, uptake of Arg and BCAA are important in maintaining homeostatic levels of other AA important for milk protein synthesis. In theory, limitation of any one of these AA could deprive the mammary gland of the resources it needs to synthesize milk proteins, resulting in reduced milk protein yield.

However, recently the basis of the limiting AA theory and the usefulness of its application to explaining milk protein production in ruminants has been questioned (Appuhamy et al., 2012). One of the reasons it has been questioned is we discovered that, in addition to serving as building blocks, AA work to regulate protein synthesis (Meijer, 2003, Proud, 2007). Amino acid uptake is regulated jointly with the demand for milk protein synthesis and the efficiency of transfer of AA into milk protein which are both variable, violating a key assumption of the theory that uptake of required nutrients is constant (Mitchell and Block, 1946). In addition, AA, such as Arg and Leu, function as signals upstream of translation regulator mTORC1, which also violates the assumption that one AA can be limiting because an individual AA has the capacity to influence milk protein production regardless of the presence of others (Hanigan et al., 2000, Wolfson and Sabatini, 2017). Thus, the current AA limitation model that is used to describe the protein requirements for lactating dairy cattle is not representative of the ongoing protein production processes at the cellular level (NRC, 2001, Appuhamy et al., 2012). This highlights the need to explicate the molecular mechanisms regulating milk protein synthesis

in response to nutrients in order to provide dietary recommendations that are reflective of the underlying molecular biology. *In vitro* studies aimed at unravelling underlying molecular workings responsible for the impact of AA on milk protein production have provided insight into the key molecular pathways involved.

Effects of amino acids on translational regulation in BMEC

In vitro techniques have been instrumental in attempting to elucidate the importance of specific AA and the consequences of their absence in lactating dairy cows. Initial studies in dispersed mammary epithelial cells demonstrated that the synthesis of milk-specific proteins increased with the addition of AA to cell culture medium (Schingoethe et al., 1967, Park and Chandler, 1976). A subsequent study demonstrated that availability of AA in bovine mammary acini medium increased milk protein synthesis by 50% within 1 h of incubation (Burgos et al., 2010). The mTORC1 pathway, which plays and important role in regulating protein synthesis, has demonstrated responsiveness to AA abundance and type. A study in bovine mammary tissue slices demonstrated that the mTORC1 pathway was sensitive to the presence of AA in culture medium (Arriola Apelo et al., 2014). In response to specific ratios of Lys, mTORC1 activity increased, as well as the expression of milk protein genes in immortalized bovine cells (Nan et al., 2014, Gao et al., 2017). Furthermore, Lys has been found to promote protein synthesis in BMEC, as its presence activated global protein synthesis through the mTORC1 pathway (Lin et al., 2018). The addition of Leu or Ile alone increased mTOR phosphorylation and casein fractional synthesis in mammary tissue slices (Appuhamy et al., 2011, Appuhamy et al., 2012). mTORC1 phosphorylation decreased by 76% when all EAA were removed from dulbecco's modified eagle medium (DMEM), a commonly used basal medium for mammalian cells (Appuhamy et al., 2012). Furthermore, the removal of Ile, Leu or Arg decreased mTORC1

phosphorylation by at least 50% in MAC-T cells, an immortalized bovine epithelial cell line (Appuhamy et al., 2012). Deprivation of all AA or Leu affected phosphorylation of mTORC1 targets S6K1 and 4EBP1, and fractional synthesis rates of whey protein β-lactoglobulin in BMEC (Moshel et al., 2006). Recently, 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-Casien synthesis (Wu et al., 2016). Although it is clear that AA have an effect on milk protein synthesis, the potency of individual AA as signalling molecules and the influence they have on the pathways regulating milk protein synthesis has yet to be elucidated. Understanding the effects of individual AA on these signalling proteins and pathways has the potential to facilitate increased N efficiency and more precise regulation of milk protein synthesis in the mammary gland of lactating dairy cows.

In response to AA deprivation, the ISR is activated, eIF2α is phosphorylated, and global protein synthesis attenuation occurs in mammalian cells (Pakos-Zebrucka et al., 2016). The inverse is also true; when AA are abundant, ISR signalling is diminished as seen in decreased eIF2α phosphorylation. Several studies have attempted to determine the role of the ISR pathway in regulating milk protein synthesis in response to AA. A study where all EAA were removed from DMEM caused the increase eIF2α phosphorylation in MAC-T cells, indicating the ISR was activated by AA deprivation (Appuhamy et al., 2011). In a subsequent study, subtraction of EAA from DMEM also caused upregulation of eIF2α in bovine mammary tissue slices (Appuhamy et al., 2012). However, in this same study they found that no individual EAA deprivation or addition alone significantly affected eIF2α phosphorylation (Appuhamy et al., 2012). Still, the supplementation of all EAA downregulates the ISR in bovine mammary cells and tissue slices (Appuhamy et al., 2011, Appuhamy et al., 2012). In contrast, a recent study

found that supplementation of Arg alone rescued milk fat and protein synthesis *in vitro* by inhibiting GCN2 activation in BMEC (Xia et al., 2016). However, the role of AA sensor GCN2 in detecting combined and individual AA deprivation has yet to be characterized in primary BMEC.

CRISPR/Cas9 Technology

CRISPR/Cas9-mediated KOs

The first experimental evidence of the role of type II Clustered regularly interspaced short palindromic repeats (**CRISPR**) as an adaptive microbial immune system came from within the dairy industry from a study of the phage defence in the bacterial strain *Streptococcus thermophilus* (Barrangou et al., 2007). This naturally occurring system, engineered to perform genome editing in mammalian cells, consists of two key components: the single guide RNA (**sgRNA**) that designates the genomic target and the endonuclease Cas9 which cleaves the targeted DNA (**Figure 2.3**). The sgRNAs designed for CRISPR knockout experiments consists of a 20 base pair guide sequence followed by a 3 base pair protospacer-adjacent motif (PAM) that is essential for Cas9 recognition and cleavage (Doench et al., 2016). Repair of the double-stranded breaks by the error-prone non-homologous end joining pathway results in Indel mutations that cause frameshifts leading to prevents the production of functional protein encoded by the targeted gene (Figure 2.3). CRISPR/Cas9 has been employed for many different applications in numerous mammalian species but has yet to be applied to help elucidate the molecular mechanisms governing milk component synthesis in dairy cattle.

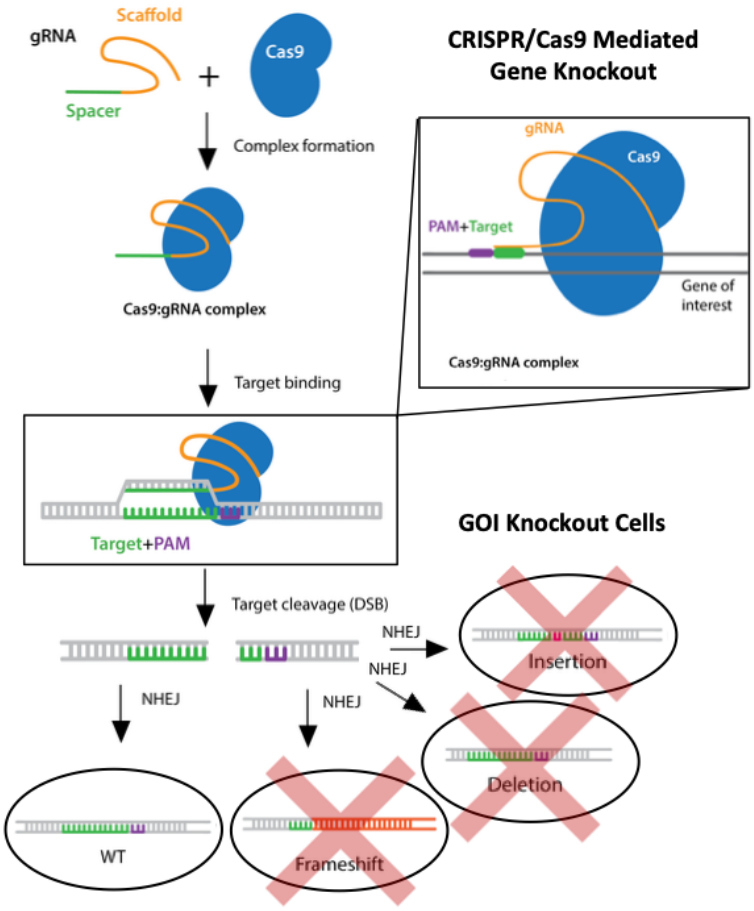

Figure 2.3. Generating CRISPR/Cas9-mediated gene knockout schematic. Upon Cas9 binding to the PAM sequence and the gRNA matching the target sequence the error prone non-homologous end joining (NHEJ) pathway is initiated. NHEJ results in small indels in the DNA that cause insertions, deletions, and frameshift mutations that ultimately result in loss-of-function mutations (Adapted from: https://www.addgene.org/guides/crispr/).

CRISPR/Cas9 in ANSC

The CRISPR/Cas9 system has revolutionized many fields but, thus far, has had limited applications in agriculture animals. CRISPR/Cas9 technology has primarily been used for the purpose of generating transgenic animals and there have been few functional gene studies. In ruminants, several studies have taken place employing CRISPR/Cas9 to examine gene function

in goats and sheep. In a study by Ni et al., 2014, four genes were disrupted in goat fibroblasts using CRISPR/Cas9**.** In this study, it was reported that CRISPR/Cas9 can induce both monoallelic and biallelic gene KO in goat primary fibroblasts. Cas9 endonuclease with specific sgRNAs targeting *MSTN*, *BLG*, *PrP*, and *NUP* genes, encoding myostatin, β-lactoglobulin, prion protein, and nucleoporin protein respectively, were simultaneously transfected into primary goat fibroblasts. Biallelic MSTN KO were then selected and used for nuclear transfer resulting in MSTN knockout goats created by targeted frameshift mutation. This study demonstrated that CRISPR/Cas9 could be effectively used to knock out genes for both functional and transgenic studies in goats by generating KO fibroblasts for target genes as well as cloned goats with biallelic MSTN mutations (Ni et al., 2014). Similarly, the *MSTN* gene was also knocked out in sheep using the CRISPR/Cas9 system coupled with zygote microinjection technology (Crispo et al., 2015). In this study, both monoallelic and biallelic lambs were generated demonstrating that the CRISPR/Cas9 KO system was also effective in sheep. Subsequently, multiplex gene editing using CRISPR/Cas9 was used to knock out three genes, *MSTN*, *ASIP* (encoding agouti signaling protein), and BCO2 (encoding β-carotene oxygenase 2), in sheep at once (Wang et al., 2016). In addition, CRISPR/Cas9 was used to induce MSTN KO in primary goat mammary epithelial cells (Tian et al., 2018). Tian et al. (2018) performed a functional gene study by transfecting goat mammary epithelial cells with CRISPR/Cas9 to investigate the role of stearoyl-CoA desaturase 1 (SCD1), which is an important player in fatty acid synthesis. In this study, CRISPR/Cas9 generated KO were used to generate monoallelic SCD1 goat mammary epithelial cell KO. These KO demonstrated that in the absence of SCD1, there was a decrease in genes involved in *de novo* were decreased as well as fatty acid transporters. In addition, there were decreases in triacylglycerol, cholesterol, and the desaturase index. This study illustrates the potential for the

expansion of CRISPR/Cas9 tools into the animal sciences for the functional studies of genes involved in the milk component synthesis.

Aside from a few select studies in ruminants, CRISPR/Cas9 has primarily been used to create transgenic animals of monogastric species, primarily pigs. In pigs, Hai et al., 2014 chose to target the *vWF* gene, important in the human disease called von Willebrand disease which lacks an adequate animal model. It is thought to play an important role in the bleeding of pigs which is important in meat production. Using the CRISPR/Cas9 system, *vWF* biallelic KO pigs were engineered through direct cytoplasmic injection of Cas9 mRNA and sgRNA into zygotes. This study demonstrated that the CRISPR/Cas9 system can be used to generate mutant KO pigs in one step through direct zygote injection. This method was also used in two subsequent studies to generate gene-modified pigs via injection of zygote with Cas9 and sgRNAs with targeting either one or three specific genomic loci (Wang et al., 2015, Wang et al., 2016). The potential application of CRISPR/Cas9 in animal health was demonstrated through the generation of gene-edited pigs that were protected from porcine reproductive and respiratory syndrome virus. In a study by Whitworth et al., 2016, CRISPR/Cas9 was used to knock out key receptor for porcine reproductive and respiratory syndrome virus CD163 which protected them from the virus. In a subsequent similar study by the same group, CRISPR/Cas9 was used to KO amino peptidase N which acts as a receptor for porcine alpha coronaviruses, transmissible gastroenteritis virus, and porcine endemic diarrhea virus (Whitworth et al., 2019). However, although the receptor was ablated this transgenic alteration only conferred resistance to transmissible gastroenteritis and not to porcine endemic diarrhea virus (Whitworth et al., 2019). These studies highlight a few of the many potential applications of CRISPR/Cas9 KO technology that have yet to be utilized in the context of the dairy industry.

Objectives

The overarching objective of this thesis was to study the arm of the ISR pathway responsible for responding to AA deprivation stress in BMEC. Our specific objectives were to 1) determine the role of GCN2 in sensing AA deprivation and 2) its capability of selectively upregulating genes involved in AA homeostasis in BMEC. Primary BMEC were selected as a model, as they are the most representative model of milk producing cells (Jedrzejczak and Szatkowska, 2014). To accomplish the first objective, we used CRISPR/Cas9 knockout gene editing in primary BMEC to ablate GCN2 and measured changes in protein abundance and phosphorylation status using immunoblotting. To accomplish our second objective, we measured changes in phosphorylation status and gene expression using immunoblotting and real time quantitative PCR in primary BMEC.

Chapter 3 – Manuscript

RUNNING HEAD: GCN2 senses amino acid deprivation in BMEC

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, S.A. Burgos

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

Corresponding author:

Sergio A. Burgos

Department of Animal Science

McGill University

21,111 Lakeshore Road,

Sainte-Anne-de-Bellevue, QC

Canada H9X 3V9

Tel.: (514) 398-7802

Fax: (514) 398-7964

E-mail: sergio.burgos@mcgill.ca

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 5-7 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 eIF2α 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 eIF2α Ser51 after 1 h of deprivation, respectively. In addition, activation of GCN2 as measured by increased phosphorylation of eIF2α Ser51 during the deprivation of Arg, Leu, and Lys combined and of Arg alone was sustained for up to 8 h of deprivation. Phosphorylated eIF2α 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 deprivation 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 RNAs targeting *EIF2AK4* were not as responsive to combined AA deprivation, compared to BMEC transduced with non-targeting single-guide RNAs. Taken together, our results demonstrate a critical role for GCN2 in the adaptive response of BMEC to AA deprivation.

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 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 in order 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 eIF2α at Ser51, a critical control point in mRNA translation, which results in general inhibition of protein synthesis (Berlanga 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 eIF2α by GCN2 promotes the selective translation of ATF4, a transcription factor that induces the expression of genes that enable adaptation to AA scarcity in order 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). There is evidence that the presence of essential AA 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 towards energy production and synthesis of non-essential AA (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, Appuhamy et al., 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 the first or second 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 non-homologous 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 activates an adaptive response mediated by GCN2 in BMEC. To

test this hypothesis, we first characterized the ISR pathway 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 activating the adaptive AA response.

MATERIALS AND METHODS

Isolation and Culture of Bovine Mammary Epithelial Cells

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× antibiotics/antimycotics (100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, and 50 μg/mL gentamicin) for transport to the laboratory. After removal of visible fat, connective tissue, and blood vessels, mammary tissue was minced into ~1 mm^3 pieces using scalpels and then rinsed 5× using ice-cold Ham's F12 medium supplemented with 1× 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× antibiotics/antimycotics at 37 °C with constant shaking (80 rpm) for 4 h. The tissue digest was filtered through a 200-μm mesh sieve and then centrifuged at 80×*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 (M2162; US Biological; Salem, MA), 0.25% (vol/vol) fetal bovine serum, 0.1% (wt/vol) Albumax II, 7.5

μg/mL bovine insulin, 0.3 μg/mL hydrocortisone, 5 ng/mL recombinant human epidermal growth factor, 2.5 μg/mL bovine apo-transferrin, 5 μ*M* isoproterenol, 5 p*M* 3,3′,5-triiodo-L-thyronine, 0.5 p*M* β-estradiol, 0.1 n*M* oxytocin, and 1× 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 ThermoFisher Scientific (Burlington, ON, Canada), unless specified otherwise.

Design and Cloning of Lentiviral Vector for CRISPR Knockout

Single guide RNAs targeting exons 1 and 4 of *EIF2AK4* (NCBI 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 sgRNAs with the highest predicted efficiency scores (Doench et al., 2016), lowest predicted off-targets (up to 3 mismatches in the protospacer), 40-60% GC content and ≤ 1 self-complementarity score. Two control sgRNAs were selected from a universal non-targeting (**NT**) control sgRNA library (Doench et al., 2016). A BLASTn search confirmed that the selected NT sgRNAs lacked sequence homology with the bovine genome (Boratyn et al., 2012). The sequences of the sgRNAs used in this study are in Table 1.

The sgRNAs were synthetized as single-stranded DNA oligonucleotides by ThermoFisher 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 sgRNAs, 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 media containing lentiviral particles was harvested, centrifuged at 800×*g* for 5 min 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 μg/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 μg/mL of puromycin (P8833; Sigma-Aldrich), which was replaced every 2 d. Transduced BMEC were grown to 50-60% confluence for collection and cryopreservation.

Genomic Cleavage Assay

The GeneArt Genomic cleavage assay detection kit (A24372; ThermoFisher 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 – fraction cleaved)$^{1/2}$).

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 μg/mL puromycin. The medium was changed every 2 d. To induce lactogenic differentiation, cells were incubated in DMEM modified to contain 3.5 m*M* D-glucose and 1 m*M* sodium acetate supplemented with lactogenic hormones (**LH**; 5 μg/mL each of bovine insulin, ovine prolactin and hydrocortisone), 5 μg/mL bovine apo-transferrin, 0.5 mg/mL BSA and 1× antibiotic-antimycotics for 5-7 d. The concentrations of AA in DMEM was (in μ*M*): Cys, 200; Gln, 4000; 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). 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). 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 µ*M* 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*, *ATCB* 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 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 1% (vol/vol) Triton X-100, 1 m*M* EDTA, 50 m*M* β-glycerophosphate, 50 m*M* NaF, 10 m*M* Na_4PO_7, 10 m*M* 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×*g*, 4 °C, for 15 min. A portion of the cleared cell lysate was combined with 5× 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 (ThermoFisher Scientific) with BSA as standard. Equal amounts of protein were resolved by SDS-PAGE and then transferred onto PVDF membranes. The membranes were blocked in 5% (wt/vol) non-fat 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% non-fat milk in TBS-T at 4 °C with constant rocking overnight. Primary monoclonal antibodies (clone name; catalogue number) used were phospho-eIF2α Ser51 (D9G8; 3398), eIF2α (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× in TBS-T for 5 min, membranes were incubated with secondary antibodies diluted 1:10,000 in 5% non-fat 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 m*M* Tris-HCl, pH 6.8, 2% (wt/vol) SDS, and 100 m*M* 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. 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 in by Degasperi et al. (2014), which are 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 square means ± SE. 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 eIF2α Ser51 and S6K1 Thr389, respectively. Compared to cells incubated in control medium containing all AA, combined deprivation of Arg, Leu, and Lys activated GCN2, as inferred by eIF2α 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 eIF2α Ser51 and S6K1 Thr389 compared to cells incubated in control medium containing all AA. Deprivation of Arg and Lys, but not Leu, induced eIF2α 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 eIF2α Ser51 phosphorylation by at least two-fold at 1, 4, and 8 h ($P < 0.001$), but not at 24 h (Figure 2A), compared to 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 to 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). *ASNS* expression 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 two independent sgRNAs targeting exon 1 and 4 of *EIF2AK4* (Figure 4A). We confirmed that BMEC transduced with sgRNAs *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 there was no detectable protein in BMEC transduced with sgRNA targeting the gene compared to 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 induce an adaptive response to AA deprivation 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 eIF2α 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).

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 immortalized cells and tissue slices (Appuhamy et al., 2012, Arriola Apelo et al., 2014). Appuhamy et al. (2012) found that no single AA influenced eIF2α phosphorylation in mammary tissue and MAC-T cells. However, there is a 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 signalling cascade over a 24 h period. We found that both the combined deprivation of AA and the individual deprivation of Arg acutely activated the ISR response. In addition, the ISR shares an endoplasmic reticulum stress sensor with the unfolded protein response, PERK. Just as GCN2 phosphorylates eIF2α during AA deprivation, PERK phosphorylates eIF2α 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 essential AA infusion into the abomasum of lactating Holstein cows, which lead to an increase in milk protein yield. In this same study, they also found that mTORC1 in the skeletal muscle responds to abomasally-infused essential AA, whereas nutritional regulation in response to essential AA 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 essential AA *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 eIF2α, which specifically upregulates ATF4 translation. ATF4 induces the expression of 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 when compared to the control containing all AA at 8 h. Likewise, there was a greater elevation of eIF2α phosphorylation at 1 h with Arg deprivation alone than with combined AA deprivation treatments when compared to respective controls, both containing all AA. 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 signalling 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 dietary essential (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 (Mepham, 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 an adaptive AA response in comparison with Leu and Lys. However, 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). Furthermore, 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 plays 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. Since estimates of Arg requirements for dairy cows are not well defined, it could be that the supply of metabolizable protein 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 bovine mammary

epithelial cells. 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 eIF2α 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 eIF2α was not affected in lactating cows given an abomasal infusion lacking Lys, compared to 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 instead of MAC-T cells, as BMEC are a more biologically relevant model (Jedrzejczak and Szatkowska, 2014).

We did not observe gross differences in growth rate between BMEC transduced with NT and *EIF2AK4* sgRNA. 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, 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 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, characterization the role of GCN2 in other cellular functions 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 activates an adaptive AA response in BMEC. Notably, our results demonstrate that the individual absence of Arg in BMEC culture medium has a more pronounced effect on eIF2α phosphorylation than the absence of Leu or Lys. In addition, we showed that both combined AA deprivation and the individual deprivation of Arg induced eIF2α Ser51 phosphorylation for up to 8 h. We discovered that *ASNS* and *SLC7A1*, downstream gene targets of ATF4, specific to adaption 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 response to AA deprivation and establishes the use of lentiviral-delivery CRISPR/Cas9 as a powerful tool to characterize gene function in primary BMEC.

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Table 3.1. List of sgRNAs used in this study

sgRNA	Sequence (5'→3')	Forward Primer (5'→3')	Reverse Primer (5'→3')	Exon	Product size (bp)
EIF2AK4_1	CACCGCACTGCTATGGCCGG	TAGTCTTCCTGCCAGAGGGA	GACCAGACATGCCAAGTTCC	1	436
EIF2AK4_2	GGAGGCAGTTAGAGGCCAAG	TTCCTGCTGTGGTTACCTCC	TCCTAGGGCCTCTTTTCACA	4	476

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Table 3.2. 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)
ACTB	NM_173979.3	GACCCAGATCATGTTCGAGA	CTCATAGATGGGCACCGTGT	449, 593	455/456	145
ASNS	NM_001075653.1	TATCCAGAGAGAGCCTGGAGC	GGACCCCTGTGTGCAATCTT	106, 223	112/113	118
GAPDH	NM_001034034.2	GAGGGGCTGCCCAGAATATC	CCAGTGAGCTTCCCGTTGAG	664, 754	742/743	91
PPIA	NM_178320.2	GGCAAGTCCATCTATGGCGA	GCCATCCAACCACTCAGTCT	234, 383	373/374	150
SLC7A1	NM_001135792.1	GGTCTTACGATACCAGCCCG	GTCTGAGAATCGCTGCTGCT	1284, 1397	1292/1293	114
SLC38A2	NM_001082424.1	TGAAGAGCTTAAAGGCCGCA	GGTATCCAAAGAGGGCAGCA	1290, 1393	1304/1305	104

Edick_Figure 1.

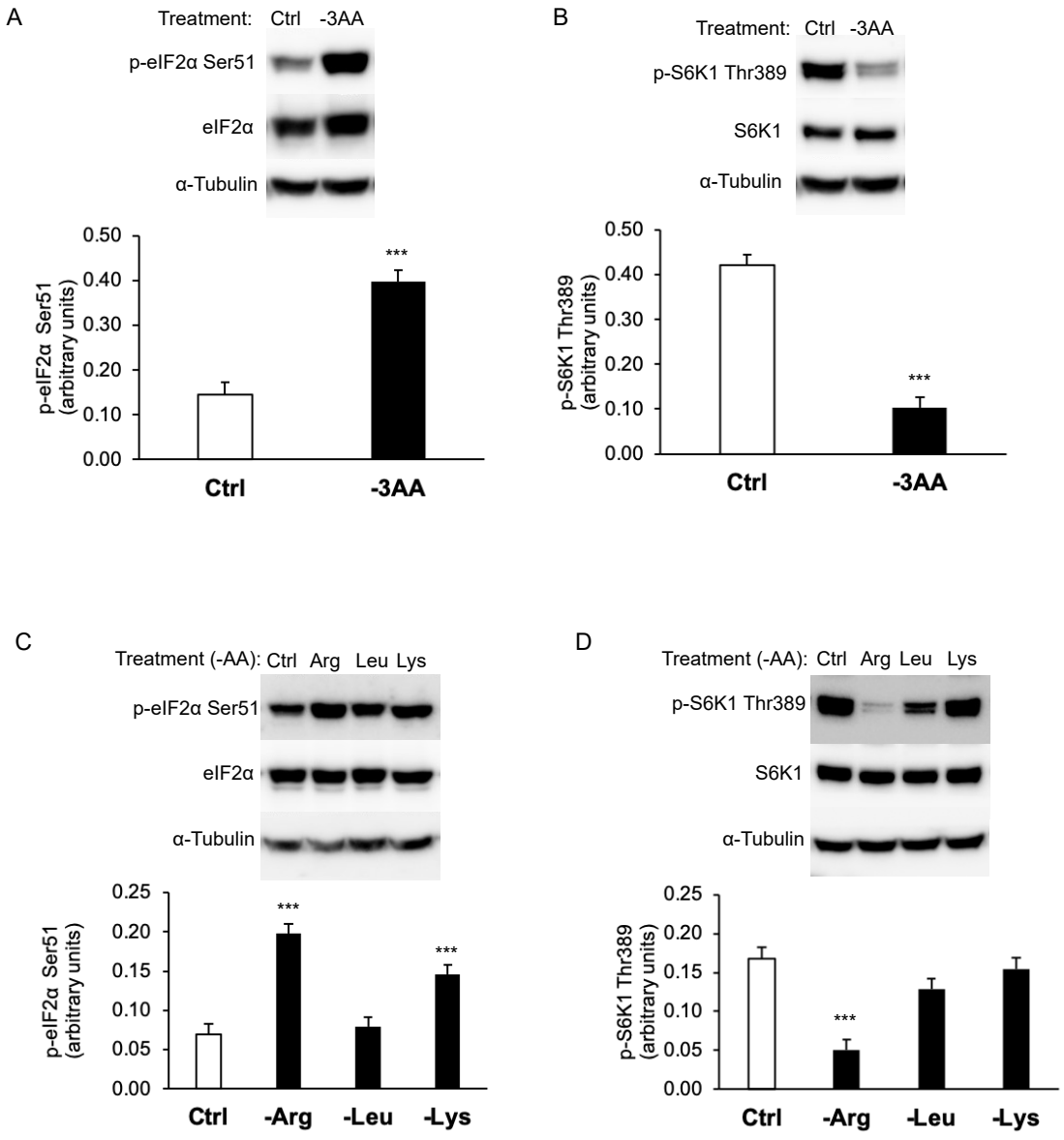

Figure 3.1. Effect of individual amino acid deprivation on ISR and mTORC1 signaling in BMEC. Lactogenic differentiated bovine mammary epithelial cells were incubated in complete medium (Ctrl) or deprived of Arg, Leu, and Lys combined (-3AA) (A and B) or alone (C and D) for 1 h. Phosphorylation (p) of (A and C) eIF2α 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 least squares means ± SE for $n = 5$ or 6. *** $P < 0.001$. Representative immunoblots are shown.

Edick_Figure_2.

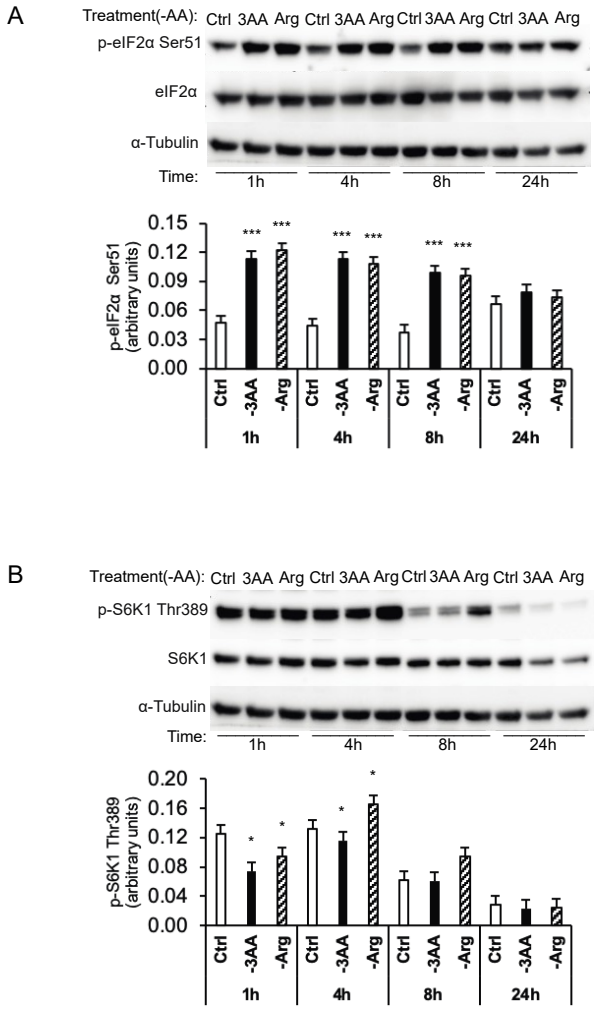

Figure 3.2. Time course of amino acid deprivation on ISR and mTORC1 in BMEC. Lactogenic differentiated bovine mammary epithelial cells were incubated in complete medium (Ctrl) or deprived of Arg, Leu, and Lys combined (-3AA) or Arg alone for 1, 4, 8, and 24 h. Phosphorylation of (A) eIF2α Ser51 and (B) 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 least squares means ± SE for $n = 3$. *** $P < 0.001$; * $P < 0.05$. Representative immunoblots are shown.

Edick_2020_Figure_3

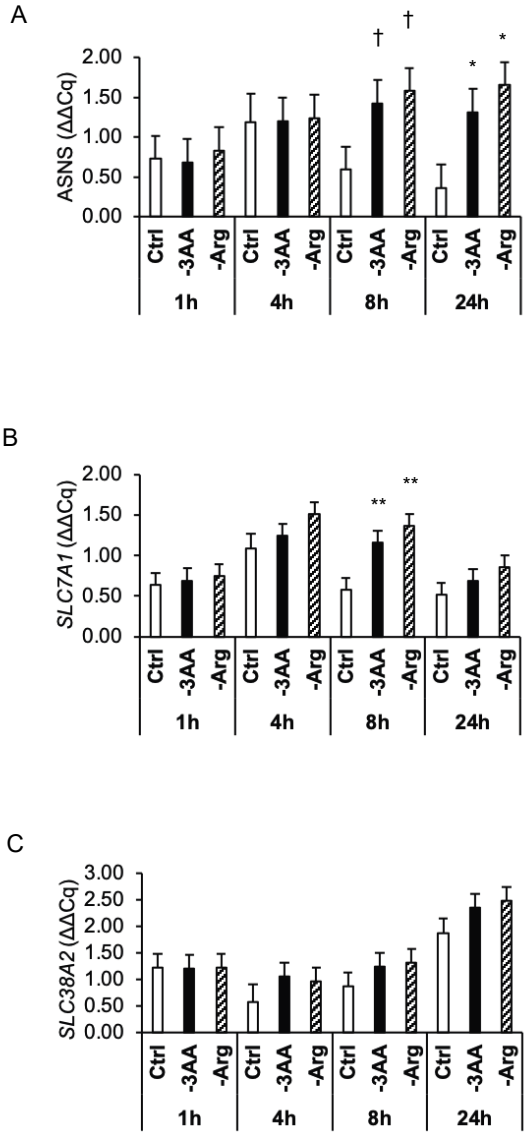

Figure 3.3. Time course of amino acid deprivation on downstream targets of the ATF4 in BMEC. Lactogenic differentiated bovine mammary epithelial cells were incubated in complete medium (Ctrl) or deprived of Arg, Leu, and Lys combined (-3AA) or Arg alone for 1, 4, 8, and 24 h. mRNA abundance of (A) *ASNS*, (B) *SLC7A1* and (C) *SLC38A2* was measured by qPCR. The

abundance of target mRNA is expressed as the normalized fold change ($\Delta\Delta C_q$). Values are least squares means ± SE for $n = 3$. ** $P < 0.01$; * $P < 0.05$; † $P < 0.10$.

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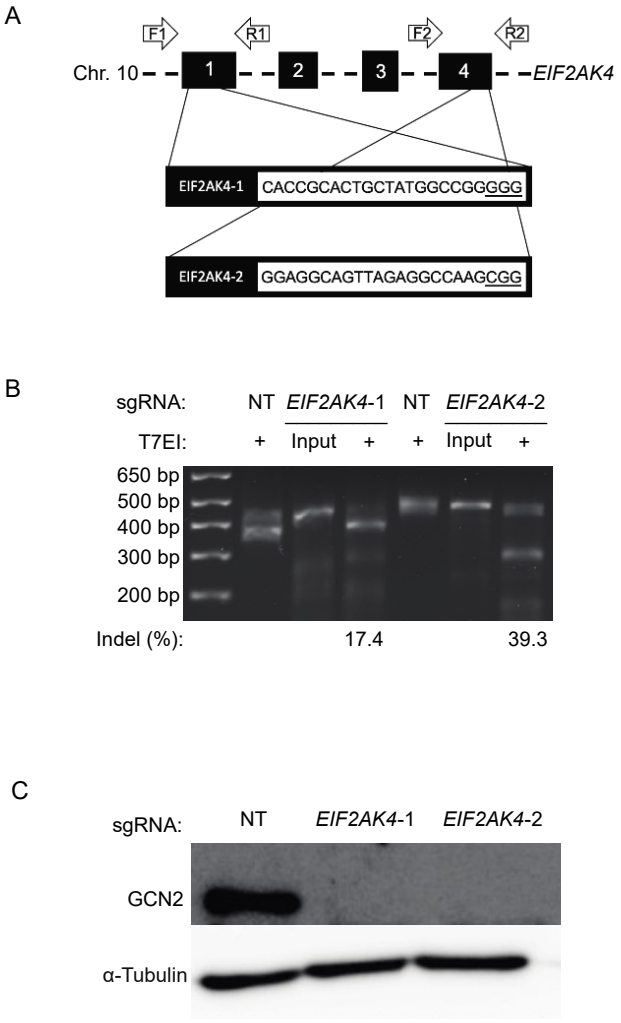

Figure 3.4. CRISPR-Cas9 mediated knockout of GCN2 in BMEC. (A) A schematic of *EIF2AK4* exons targeted by the single guide RNA (sgRNA). The sequence targeted by each sgRNA is shown with the protospacer adjacent motif underlined. The relative position of the primers for genomic cleavage are shown. (B) 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 bovine mammary

epithelial cells transduced with non-targeting 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.

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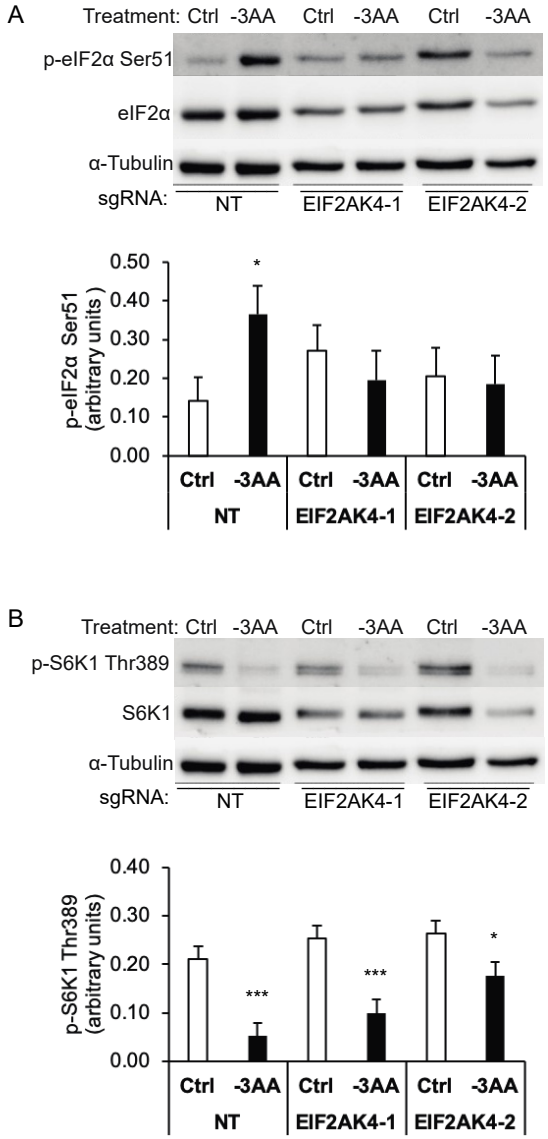

Figure 3.5. Effect of amino acid deprivation on the ISR and mTORC1 pathways in GCN2 knockout BMEC. Lactogenic differentiated GCN2 knockout bovine mammary epithelial cells transduced with non-targeting (NT) control sgRNA or sgRNA targeting the coding region of *EIF2AK4* (*EIF2AK4*-1 and *EIF2AK4*-2) were deprived of Arg, Leu, and Lys combined (-3AA) or incubated in complete medium (Ctrl) for 1 h. Phosphorylation (p) of (A) eIF2α 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 least squares means ± SE for n = 5 or 6. *** $P < 0.001$; * $P < 0.05$. Representative immunoblots are shown.

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Chapter 4 – General Discussion

Our understanding of AA nutrition for lactating dairy cows has been significantly shaped by the prevailing limiting AA theory that suggests the AA in the shortest supply limits protein synthesis (NRC, 2001). *In vivo* studies focused on Met, Lys, Arg and Leu have produced inconsistent results that do not support this theory. *In vitro* experiments have shed light on key pathways at the level of the regulatory complexes involved in milk protein synthesis. However, they too produced variable results when assessing the effects of specific AA including Met, Lys, Arg, and Leu. Despite new developments in our understanding of AA as signalling molecules and the discovery of specific upstream AA sensors in other mammalian cell types (Wolfson and Sabatini, 2017), little research has been done to determine the effects of these sensors on milk protein production or to produce updated theories on the regulation of milk protein production by AA.

The view of AA as simply building blocks for milk protein production is expanding to include the role of AA as signalling molecules. It is known that specific AA such as Arg and Leu signal sensors upstream of complexes that regulate protein synthesis activity in mammalian cells. This development in our understanding of AA highlights the importance of characterizing the sensors of AA status. This is of particular importance to sensors that are part of pathways regulating milk protein production in BMEC. Accumulated research has shown that both the ISR and mTORC1 are important pathways in regulating protein synthesis in response to AA in bovine mammary tissue and cells. It has shown that AA that are taken up by the mammary gland in excess of requirements needed for milk protein production (Manjarin et al 2014), such as Arg, Leu, and Lys, are important in regulating milk protein synthesis through these two key pathways. However, the focus of research has been on the main kinases, eIF2α and mTORC1; the role of

upstream AA sensors upstream of these pathways have yet to be elucidated. We sought to determine the role of the ISR AA deprivation sensor GCN2 in response to specific AA deprivation in several contexts.

Our research demonstrated the importance of GCN2 in sensing AA deprivation and initiating adaptive action to restore AA homeostasis in BMEC. In particular, our results showed that the absence of Arg appears to have a unique role in signalling AA deficiency through GCN2 and target kinase eIF2α. This is a surprising outcome as GCN2 senses AA status through uncharged tRNA which should not discriminate between the absence of one AA over another. Furthermore, this could indicate that there are other AA that demonstrate an increased capacity for signalling AA deficiency and triggering subsequent adaptive action. The absence of Arg was also the only individual AA to effect mTORC1 as seen through S6K1 signalling once again suggesting that Arg may stand apart from other AA in regulating protein translation.

There were some limitations to our study. Although we demonstrated that Arg affected translational regulators, we did not measure protein synthesis as our focus was on the sensing by GCN2 and activation of the ISR pathway. Further research is needed to investigate the effect of Arg deprivation on milk protein synthesis *in vitro*. Furthermore, although *in vitro* functional perturbation studies are useful in identifying the role of specific molecular elements in milk protein synthesis, *in vivo* studies are needed to characterize the role of GCN2 and the ISR in response to Arg deprivation in lactating dairy cows. Moreover, besides measuring milk protein production *in vivo*, it would be interesting to see if the deprivation of Arg affected other important milk components such as milk fat quantity and composition. Determining the role of the ISR in mediating conditions of AA deprivation *in vivo* would be an important step in elucidating the signalling pathways that influence the efficiency of milk protein production in

response to AA. Our study has provided an important first step, as well as valuable insight into the role of GCN2 in sensing AA deprivation, and is the first to employ the revolutionary CRISPR/Cas9 technology in primary BMEC.

This work has shown that CRISPR/Cas9 can be used to create a gene KO in primary BMEC providing a valuable tool for fundamental research in animal science. By using CRISPR/Cas9 to KO GCN2, we have established a role for this sensor in the sensing and activating an adaptive response to AA deficiency in BMEC. This demonstrates just one of the many potential applications for the CRISPR/Cas9 technologies in the field of animal science and provides framework for similar functional studies. The ability of CRISPR/Cas9 to efficiently introduce changes into the genome has already revolutionized other scientific disciplines. The precise generation of CRISPR/Cas9 KOs can be used to determine the function of molecular mechanisms and regulatory networks that have not yet been characterized in agricultural species. More immediately, CRISRP/Cas9 KO technology could be used to characterize other upstream AA sensors to determine the effects of specific AA on initiating or suppressing translation in BMEC. In addition to single gene KO studies, in the future, CRISPR/Cas9 can be used to KO multiple genes of interest in agricultural animal cells in targeted screens. CRISPR/Cas9 KO screens have been employed in other fields in order to characterize regulatory networks responsible for physiological processes and diseases (Shalem et al 2014; Chen et al 2015). In sum, CRISPR/Cas9 KO technology provides a precise and powerful tool for identifying and characterizing important molecular signalling pathways in agricultural species.

Gene editing technologies that allow the investigation of the molecular mechanisms underpinning protein production can assist in optimizing protein production efficiency to serve the growing population. Agricultural animals play a critical role in meeting human nutritional

requirements providing 39% of protein consumed (FAO 2018). Several research groups focussed on different livestock species have demonstrated the applications to generating CRISPR/Cas9 KO transgenic species in goats, sheep, and pigs by examining a variety of questions some of which are related to protein production (Bishop and Van Eenennaam, 2020). So far, these studies show promise in using functional gene editing to identify gene function and characterize causal gene variants. However, CRISPR/Cas9 KO technology has not been used to improve our understanding of molecular pathways governing milk protein synthesis in BMEC or dairy cattle. Use of CRISPR/Cas9 in BMEC and dairy cattle and could provide a more holistic characterization of the role of specific genes and molecular pathways involved in milk protein synthesis.

Improving our understanding of the relevant underlying genes and molecular pathways that govern milk protein synthesis in response to AA would allow us to develop the AA specificity of dietary models for lactating dairy cows. Dietary models that are optimized for AA requirements may have reduced protein requirements with equal milk protein yield. The reduction of protein in the diet of lactating dairy cattle reduces excreted N and the associated environmental consequences (Kalscheur et al 2006). Our work showed that CRISPR/Cas9 can be used to identify important molecular elements and pathways in milk protein synthesis that are responsive to specific AA. This knowledge of the underlying molecular pathways regulating milk protein synthesis serves as an important first step in employing new, powerful gene-editing technologies to improve our understanding of the underlying molecular biology governing milk protein synthesis. The knowledge from this work can be used to inform and guide future *In vivo* studies that lead to the optimization of N utilization through improved dietary recommendations and reduction of the environmental pollution caused by the dairy industry.

Chapter 5 – Conclusion

This study uncovers a critical role for GCN2 in sensing AA deprivation in primary BMEC. GCN2 is important in both sensing AA deprivation and activating the ISR pathway that is responsible for taking adaptive action to restore AA homeostasis, in primary BMEC. We found that the combined deprivation of Arg, Leu and Lys activated the ISR, through GCN2, as measured by eIF2α Ser51 phosphorylation. Surprisingly, Arg deprivation in particular increased activation of the ISR through GCN2. Furthermore, we established that GCN2 was necessary for activation of the ISR by generating CRISPR/Cas9 GCN2 KO BMEC. Our work is the first to employ CRISPR/Cas9, through lentiviral delivery, to interrogate gene function in primary BMEC. It contributes original methodology for conducting CRISPR/Cas9 KO studies in primary BMEC, that can be applied to other important questions in animal agriculture, to the field of animal science. In summary, this work demonstrates the crucial role of GCN2 in sensing AA deprivation using CRISPR/Cas9 KO technology in BMEC.

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