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RESEARCH ARTICLE

Biphasic regulation of glutamine consumption by WNT during osteoblast differentiation

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ABSTRACT

Osteoblasts are the principal bone-forming cells. As such, osteoblasts have enhanced demand for amino acids to sustain high rates of matrix synthesis associated with bone formation. The precise systems utilized by osteoblasts to meet these synthetic demands are not well understood. WNT signaling is known to rapidly stimulate glutamine uptake during osteoblast differentiation. Using a cell biology approach, we identified two amino acid transporters, γ(+)-LAT1 and ASCT2 (encoded by *Slc7a7* and *Slc1a5*, respectively), as the primary transporters of glutamine in response to WNT. ASCT2 mediates the majority of glutamine uptake, whereas γ(+)-LAT1 mediates the rapid increase in glutamine uptake in response to WNT. Mechanistically, WNT signals through the canonical β-catenin (CTNNB1)-dependent pathway to rapidly induce *Slc7a7* expression. Conversely, *Slc1a5* expression is regulated by the transcription factor ATF4 downstream of the mTORC1 pathway. Targeting either *Slc1a5* or *Slc7a7* using shRNA reduced WNT-induced glutamine uptake and prevented osteoblast differentiation. Collectively, these data highlight the critical nature of glutamine transport for WNT-induced osteoblast differentiation.

This article has an associated First Person interview with the joint first authors of the paper.

KEY WORDS: WNT, β-catenin, Glutamine, Osteoblast, *Slc1a5*, *Slc7a7*

INTRODUCTION

Osteoblasts are the primary bone forming cell responsible for producing and secreting type I collagen and other proteins that comprise the bone matrix. A constant supply of amino acids is required to maintain high rates of protein and matrix synthesis associated with bone anabolism. To fulfill this demand, osteoblasts must maximize the production or acquisition of amino acids. Indeed, recent evidence links amino acid uptake and metabolism to osteoblast function (Elefteriou et al., 2006; Karner et al., 2015; Rached et al., 2010; Yu et al., 2019). However, little is known about the transporters mediating amino acid uptake, nor their regulation during bone development.

The WNT family of secreted glycoproteins are critical regulators of osteoblast differentiation (Babij et al., 2003; Bennett et al., 2005; Day et al., 2005; Gong et al., 2001; Hu et al., 2005; Rodda and McMahon, 2006; Tu et al., 2007). WNTs activate multiple intracellular signaling cascades to induce osteoblast differentiation and modulate osteoblast activity. In the canonical pathway, WNT regulates the stability of the transcriptional coactivator β-catenin (CTNNB1), a critical regulator of osteoblast specification and differentiation (Day et al., 2005; Hu et al., 2005; MacDonald and He, 2012; Rodda and McMahon, 2006). In the absence of WNT, β-catenin is phosphorylated by the β-catenin destruction complex and targeted for proteasomal degradation. WNT stimulation inhibits the destruction complex, resulting in stabilization of β-catenin, which can then translocate into the nucleus and induce expression of target genes (for example, *Tcf7*) (Angers and Moon, 2009; Clevers, 2006; Clevers and Nusse, 2012; Roose et al., 1999). WNTs can also regulate osteoblast differentiation independently of β-catenin through the serine/threonine kinase mechanistic target of rapamycin (mTOR) (Chen et al., 2014; Inoki et al., 2006; Karner et al., 2015). Indeed, mTORC1 is critical for preosteoblasts to increase protein synthesis and differentiate into mature osteoblasts (Chen and Long, 2015; Fitter et al., 2017; Karner et al., 2017; Lim et al., 2016; Xian et al., 2012). We previously discovered that WNT stimulates glutamine uptake and catabolism necessary for osteoblast differentiation and bone formation (Karner et al., 2016, 2015). Mechanistically, it is not clear how WNT stimulates glutamine uptake in osteoblasts.

Glutamine transport is facilitated by a diverse array of membrane-tethered amino acid transporters categorized into discrete transport systems based on substrate specificity, kinetics and ion and pH dependence (Pochini et al., 2014). Glutamine uptake can occur in a Na^+-dependent or -independent manner mediated by Systems ASC, A, γ(+)-L and N, or System L, respectively (Biltz et al., 1983; Bode, 2001; Jacob et al., 1986; Mackenzie et al., 2003; Tamarappoo et al., 1992, 1997; Taylor et al., 1992). Na^+-dependent transport is subdivided by the strict requirement of Na^+ [Systems ASC, A and γ(+)-L] or the substitution of Li^+ for Na^+ (System N). Finally, inhibitors can further subdivide Na^+-dependent transporters, with System A being sensitive to 2-(methylamino)isobutyric acid (MeAIB) and System ASC to γ-glutamyl-*p*-nitroanilide (GPNA) (Esslinger et al., 2005; Freeman et al., 1999; Mackenzie et al., 2003). Although a great deal is known about the functional characteristics of these transporter systems, little is known about the specific transporters mediating glutamine uptake or their regulation in osteoblasts.

Here, we describe the biphasic regulation of glutamine uptake in response to WNT. WNT rapidly stimulates glutamine uptake that is sustained throughout osteoblast differentiation. Mechanistically, WNT activates β-catenin to rapidly stimulate glutamine uptake through *Slc7a7*, whereas mTORC1 regulates basal glutamine uptake through *Slc1a5*. These data highlight previously unknown

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roles for the amino acid transporters encoded by *Slc7a7* and *Slc1a5* and their regulation by WNT during osteoblast differentiation.

RESULTS

WNT rapidly stimulates glutamine uptake during osteoblast differentiation

We previously determined that WNT stimulates glutamine uptake associated with osteoblast differentiation (Karner et al., 2015). In order to understand how WNT regulates glutamine consumption, we first evaluated the kinetics of glutamine uptake in ST2 cells, a bone marrow-derived cell line that undergoes osteoblast differentiation in response to WNT (Bennett et al., 2005; Kang et al., 2007; Karner et al., 2016, 2015; Otsuka et al., 1999; Tu et al., 2007) (Fig. 1A). ST2 cells were treated with recombinant WNT3a (rWNT3a) for up to 96 h, and glutamine uptake was quantified using L-(2,3,4-^{3}H)-glutamine. We observed a rapid and sustained increase in glutamine consumption beginning as rapidly as 6 h after rWNT3a stimulation (Fig. 1B). Glutamine consumption continued to increase throughout the course of the experiment, even 96 h after rWNT3a stimulation (Fig. 1B). Basal glutamine transport occurred primarily in a Na^+-dependent manner that could not be rescued by Li^+ or inhibited by the amino acid analog MeAIB in ST2 cells (Fig. 1C). These data indicate that glutamine uptake occurs primarily in a Na^+-dependent manner characteristic of the amino acid transport systems System ASC and System γ(+)-L (Fig. 1D). Likewise, WNT-stimulated glutamine uptake in ST2 cells was Na^+ dependent and insensitive to either MeAIB or Li^+ rescue (Fig. 1C). These data indicate that WNT-induced glutamine uptake is not the result of enhanced activity of other transport systems, rather it results from increased System ASC and/or γ(+)-L activity (Fig. 1D) (Jacob et al., 1986; Tamarappoo et al., 1997; Taylor et al., 1992).

Slc1a5 and *Slc7a7* regulate basal and WNT stimulated glutamine uptake

To determine how WNT signaling regulated glutamine uptake, we first evaluated the mRNA expression of genes encoding glutamine transporters in ST2 cells. To do this, we analyzed our previously generated RNAseq dataset of ST2 cells treated with rWNT3a for up to 72 h (Karner et al., 2016). In unstimulated ST2 cells, the System ASC transporter alanine serine cysteine transporter 2 (ASCT2, encoded by *Slc1a5*) was the highest expressed glutamine transporter in either System ASC or γ(+)-L (Table 1). Conversely, the System γ(+)-L transporter γ(+)-L transporter 1 [γ(+)-LAT1, encoded by *Slc7a7*] was expressed at low levels in unstimulated ST2 cells (Table 1). It is important to note that ASCT2 and γ(+)-LAT1 are the only members of their respective transport systems that accept glutamine as a substrate. Despite being expressed at lower levels in unstimulated ST2 cells, *Slc7a7* was significantly increased by WNT treatment at 6, 24 and 72 h (Table 1). On the other hand, expression of *Slc1a5* was not significantly increased until after 72 h WNT treatment (Table 1). We confirmed the temporal regulation of both *Slc1a5* and *Slc7a7* using reverse transcription qPCR (RT-qPCR). Both *Slc7a7* and *Slc1a5* were significantly induced by WNT signaling, albeit with differing kinetics (Fig. 2A). *Slc7a7* was induced rapidly, with peak induction occurring within 6–24 h of WNT stimulation. By 96 h, *Slc7a7* had returned to baseline expression (Fig. 2A). Conversely, *Slc1a5* was significantly induced beginning 72 h after WNT stimulation (Fig. 2A). These data demonstrate that WNT regulates two amino acid transporters with differing kinetics in ST2 cells.

We next sought to determine the necessity of these amino acid transporters for glutamine uptake. We first evaluated the role of *Slc1a5* in glutamine uptake because it was highly expressed in ST2 cells (Table 1). To do this, we used GPNA, a specific inhibitor of *Slc1a5*/ASCT2 (Esslinger et al., 2005). GPNA treatment reduced basal glutamine uptake by 77% indicating the majority of glutamine uptake is mediated by *Slc1a5*/ASCT2 (Fig. 2B). We next sought to determine the effects on WNT-induced glutamine uptake. Interestingly, despite significantly reducing basal glutamine uptake, glutamine consumption was stimulated equally by WNT in the presence or absence of GPNA (Fig. 2B,C). To confirm the specificity of GPNA, we targeted *Slc1a5* using shRNA. This

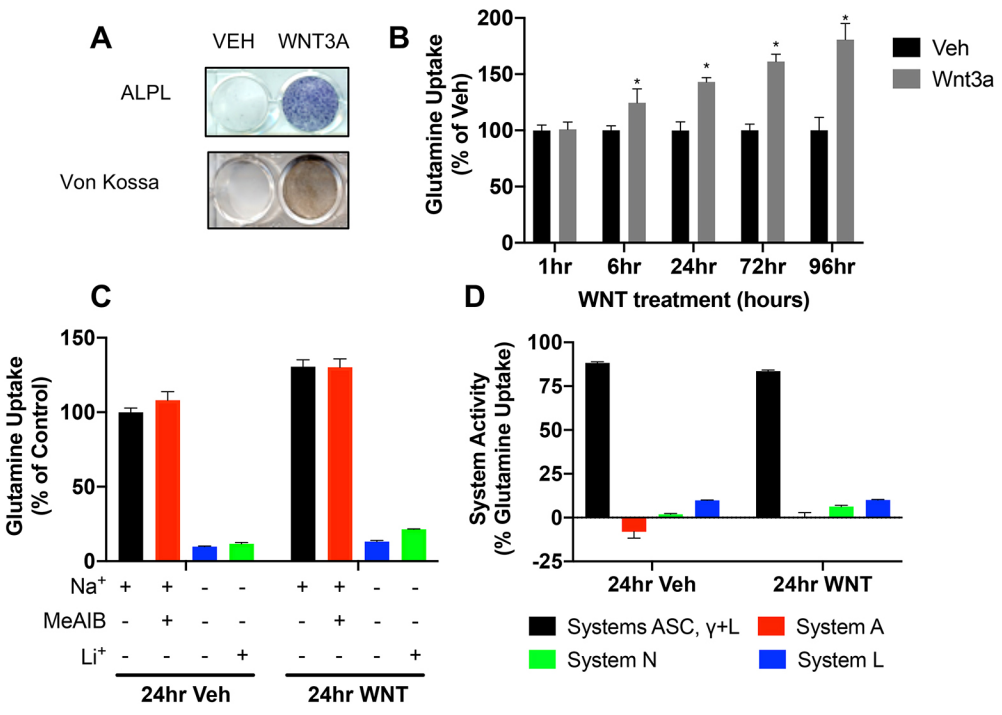

Fig. 1. WNT signaling rapidly increase Na^+-dependent glutamine consumption in ST2 cells. (A) Alkaline phosphatase (ALPL) and von Kossa staining in ST2 cells treated with 25 ng/ml rWnt3a or vehicle (VEH) for 72 h followed by osteogenic medium for 6 days. (B) Timecourse of radiolabeled glutamine uptake in response to rWNT3A. *n*=6 independent trials for each time point. (C) Glutamine uptake assays performed in the presence (+) or absence (–) of sodium (Na^+), MeAIB or lithium (Li^+) in the uptake media in ST2 cells treated with rWNT3A or vehicle for 24 h. *n*=4 for each condition. (D) Relative system activity calculated from experiments in C. Graphs depict mean±s.d. **P*≤0.05 (unpaired two-tailed Student's *t*-test).

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Table 1. Amino acid transporter expression in ST2 cells

Gene	System	Alias	FPKM[a] Control	Fold change 6 h WNT	24 h WNT	72 h WNT
Slc1a5	ASC	ASCT2	35.3	1.1	0.9	3.0*
Slc7a7	γ(+)-L	γ(+)-LAT1	1.1	5.7*	4.6*	3.8*

[a]Fragments per kilobase of transcript per million mapped reads.
*$P \leq 0.05$.

approach significantly reduced both ASCT2 protein and basal glutamine uptake, similar to the effects of GPNA treatment (Fig. 2D,E). Similar to the results of the GPNA experiment, glutamine uptake was stimulated equally by WNT in both control and ASCT2-knockdown cells (Fig. 2E,F). These data indicate that *Slc1a5*/ASCT2 mediates the majority of glutamine uptake in ST2 cells but is not responsible for the acute increase in glutamine consumption in response to WNT. We next sought to determine whether *Slc7a7* mediates glutamine uptake in ST2 cells. To do this, we targeted *Slc7a7* using shRNA. This approach effectively reduced γ(+)-LAT1 protein levels (Fig. 2G). *Slc7a7* knockdown minimally affected basal glutamine uptake but completely abrogated increased glutamine consumption in response to WNT stimulation (Fig. 2H,I). Collectively these data indicate WNT signaling rapidly increases *Slc7a7* expression to increase glutamine uptake whereas *Slc1a5* is responsible for the majority of glutamine uptake in ST2 cells.

Glutamine uptake and *Slc7a7* are regulated by canonical WNT signaling

We next sought to understand how WNT signaling regulated *Slc7a7* expression. The rapid induction kinetics of *Slc7a7* expression suggested it may be regulated directly by β-catenin. Inhibition of LRP5 and LRP6 using recombinant DKK1 (rDKK1) reduced WNT-induced β-catenin stabilization and prevented the induction of alkaline phosphatase (Fig. 3A). Moreover, DKK1 treatment prevented WNT induction of known β-catenin target genes (e.g. *Tcf7*) as well as of *Slc7a7* (Fig. 3B,C). Expression of a dominant-negative form of TCF4 that is unable to interact with β-catenin also prevented induction of *Slc7a7*, suggesting that *Slc7a7* is a β-catenin target gene (data not shown). To test the role of β-catenin directly, we targeted the gene encoding β-catenin (*Catnnb1*) using shRNA (Fig. 3D). β-catenin knockdown prevented osteoblast differentiation, as shown by inhibition of alkaline phosphatase induction (Fig. 3D). Importantly, β-catenin knockdown significantly reduced the basal expression of *Slc7a7* and prevented the induction of *Slc7a7* in response to WNT treatment (Fig. 3E). Moreover, β-catenin knockdown reduced WNT-induced glutamine uptake without affecting *Slc1a5* expression (Fig. 3F and data not shown). These data indicate that β-catenin is necessary for the induction of *Slc7a7* and acute glutamine consumption in response to WNT. We next sought to determine the sufficiency of β-catenin by inhibiting GSK3β using LiCl (Klein and Melton, 1996). LiCl treatment stabilized

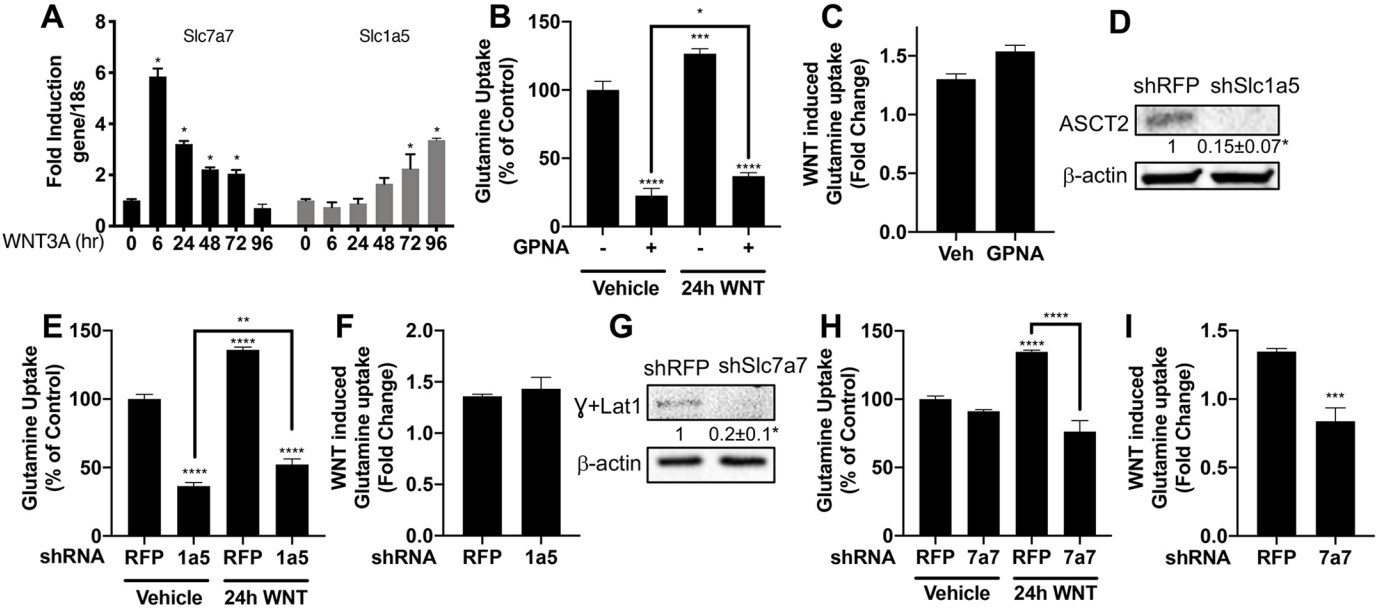

Fig. 2. Wnt signaling induces *Slc7a7* and *Slc1a5* with disparate kinetics. (A) RT-qPCR analyses of *Slc7a7* and *Slc1a5* expression in ST2 cells treated with WNT3a for up to 96 h. Fold induction±s.d. relative to 0 h time point for three independent experiments. (B) Measurements of radiolabeled glutamine uptake in response to WNT3a in the presence of GPNA. (C) Graphical depiction of WNT-induced glutamine uptake calculated from data shown in B (Veh, vehicle). (D) Western blotting analyses of ASCT2 protein expression level in ST2 cells infected with shRNA targeting *Slc1a5* or RFP as a control. ASCT2 normalized to β-actin. Fold change±s.d. relative to shRFP control is shown for three independent experiments. (E,F) Effect of *Slc1a5* knockdown (1a5) on glutamine uptake in response to WNT3a. (G) Western blotting analyses of γ(+)-LAT1 protein expression in ST2 cells infected with shRNA targeting *Slc7a7* or RFP as a control. γ(+)-LAT1 normalized to β-actin. Fold change±s.d. relative to shRFP control is shown for three independent experiments. (H,I) Effect of *Slc7a7* knockdown (7a7) on glutamine uptake in response to WNT3a. Three independent trials were performed in each experiment. *$P \leq 0.05$, **$P \leq 0.005$, ***$P \leq 0.0005$, ****$P \leq 0.00005$, by one-way ANOVA with Tukey's post hoc test (B,E,H) or by an unpaired two-tailed Student's *t*-test (A,C,D,F,G,I).

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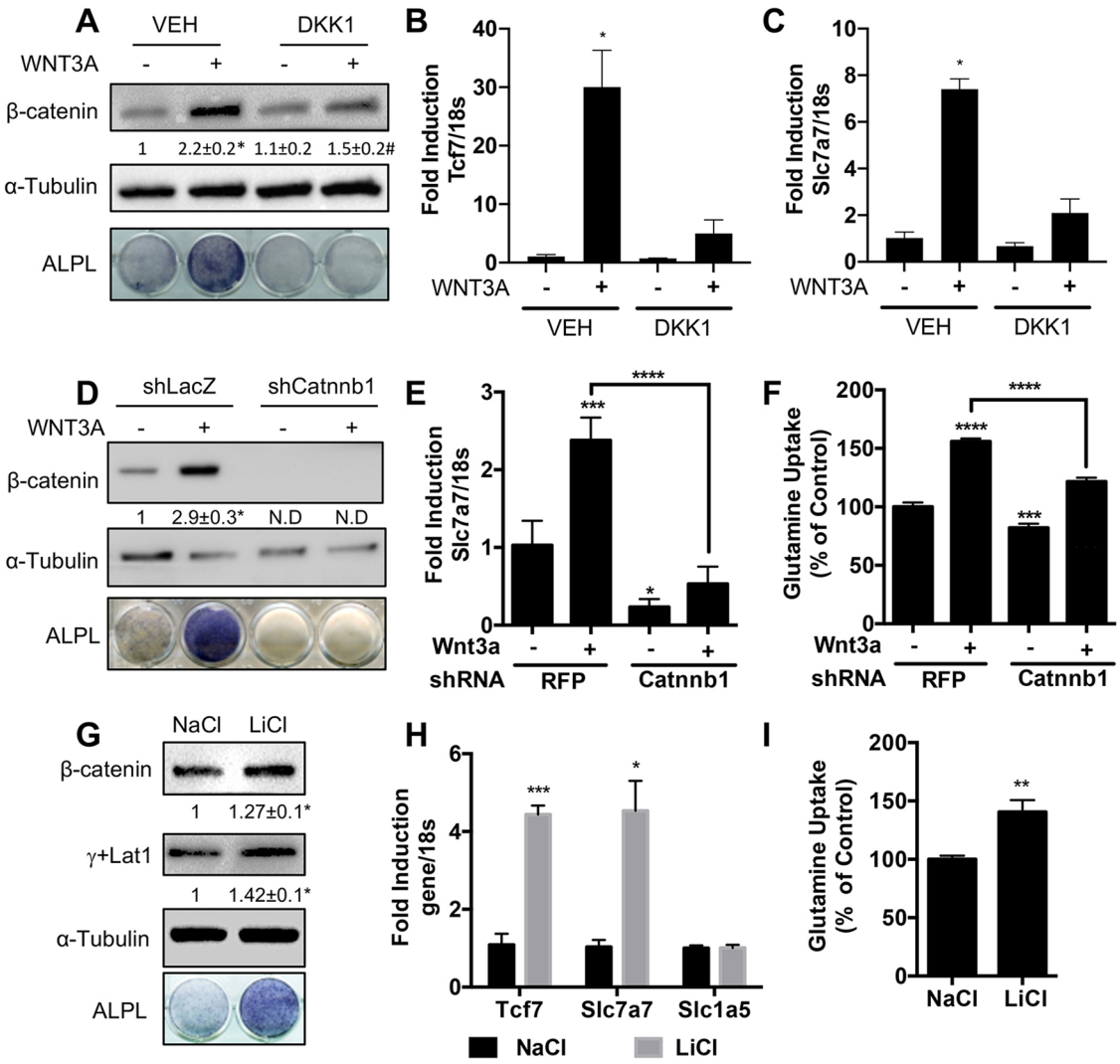

Fig. 3. *Slc7a7* is regulated by canonical WNT signaling. (A–C) Effect of rDKK1 treatment on WNT3a-induced β-catenin expression and alkaline phosphatase (ALPL) staining (A) or *Tcf7* (B) and *Slc7a7* (C) gene expression in ST2 cells, compared with vehicle-treated controls (VEH). (D–F) Effect of β-catenin knockdown (shCatnnb1) on WNT3a-induced β-catenin expression and alkaline phosphatase staining (D), *Slc7a7* gene expression (E), or glutamine uptake (F), compared with the effect of control shRNA treatment (shLacz, shRFP). (G–I) Effect of LiCl treatment on protein expression, alkaline phosphatase staining (G), gene expression (H) and glutamine uptake (I). In A,D,G, β-catenin and γ(+)-LAT1 levels are normalized to α-tubulin, and fold change±s.d. relative to untreated negative control samples are shown for three independent experiments (N.D., not determined). Data in B,C,E,F,H,I are mean±s.d. of three independent experiments. *$P \leq 0.05$, #$P \leq 0.05$; **$P \leq 0.005$, ***$P \leq 0.0005$, ****$P \leq 0.00005$ [one-way ANOVA with Tukey's post hoc test (A–F) or unpaired two-tailed Student's *t*-test (G–I)].

β-catenin and induced both alkaline phosphatase staining and *Tcf7* mRNA expression similar to WNT treatment (Fig. 3G,H). Moreover, LiCl stimulated both *Slc7a7* mRNA and γ(+)-Lat1 protein expression and increased glutamine uptake similar to WNT treatment (Fig. 3G–I). Importantly, LiCl did not increase *Slc1a5* mRNA expression (Fig. 3H). These data indicate that β-catenin-dependent WNT signaling rapidly stimulates glutamine uptake via transcriptional upregulation of *Slc7a7*.

Basal glutamine uptake and *Slc1a5* expression is regulated by mTORC1 signaling

We next evaluated the regulation of *Slc1a5* expression by WNT. Unlike *Slc7a7* and other canonical WNT target genes, which are rapidly induced by WNT, *Slc1a5* mRNA levels were not significantly increased until 72 h after WNT treatment (Fig. 2A and Table 1). Indeed, we observed no change in *Slc1a5* expression in β-catenin-knockdown cells or in response to LiCl treatment, suggesting *Slc1a5* is not regulated by canonical WNT–β-catenin signaling (Fig. 3H and data not shown). Rather, the induction of *Slc1a5* followed a timecourse reminiscent of protein anabolism genes induced by WNT through secondary activation of the transcription factor ATF4 (Karner et al., 2015). We have previously determined that WNT signals through mTORC1 to activate ATF4 and protein anabolism genes during osteoblast differentiation (Karner et al., 2015). Here, we observed a significant increase in ATF4 protein expression after 72 h of WNT treatment (Fig. 4A). Inhibition of mTORC1 signaling using rapamycin prevented ATF4 protein induction by WNT (Fig. 4A). Importantly, rapamycin treatment reduced ASCT2 expression in both unstimulated and WNT treated ST2 cells (Fig. 4A). Consistent with decreased ASCT2 expression, rapamycin significantly reduced glutamine uptake under both basal conditions and in response to WNT stimulation (Fig. 4B). Despite a significant reduction in overall glutamine uptake, WNT was able to stimulate glutamine consumption in the

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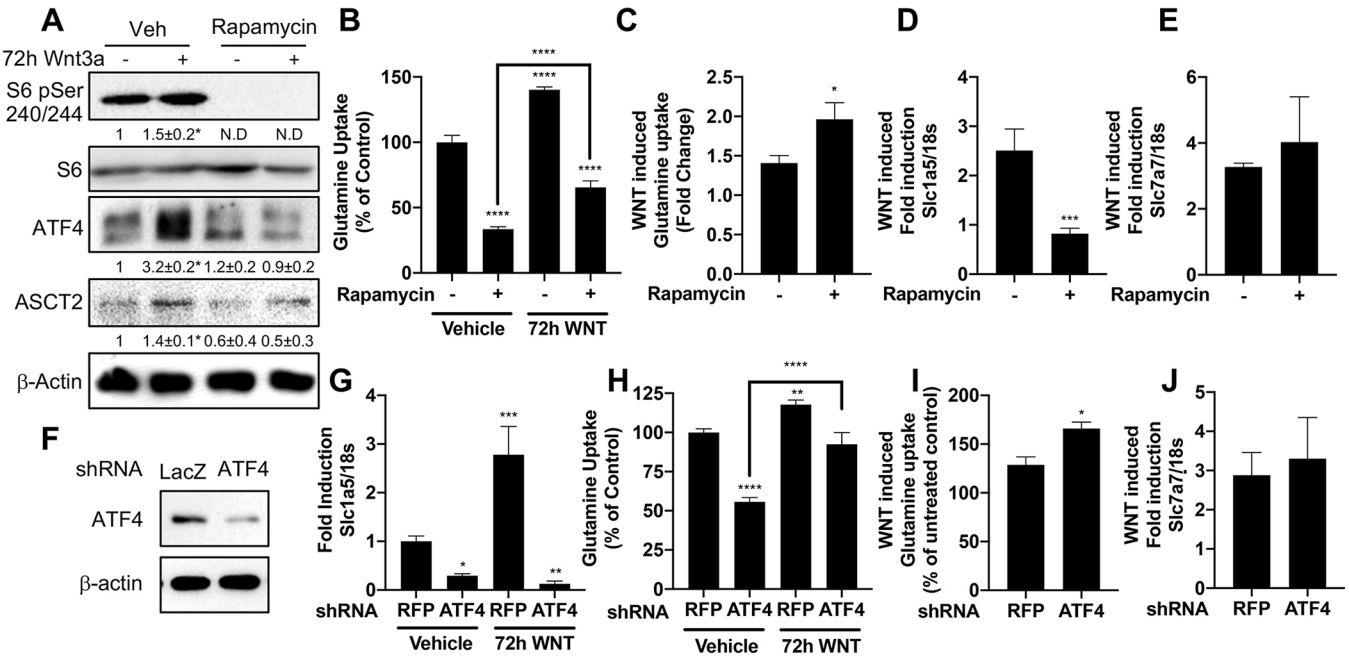

Fig. 4. *Slc1a5* is regulated by non-canonical WNT signaling. (A–D) Effect of rapamycin treatment on WNT3a-induced protein expression (A), glutamine uptake (B,C) or mRNA expression (D,E). In A, S6 phosphorylation at Ser240/244 (S6 pSer240/244) is shown as a positive control for rapamycin treatment. pSer240/244 S6 normalized to total S6, ATF4 and ASCT2 normalized to β-actin. Fold change±s.d. relative to untreated negative control samples are shown, calculated from three independent experiments (N.D., not determined). In B, data are glutamine uptake as mean±s.d. relative to vehicle-treated negative control samples, calculated from three independent experiments. In C–E, data are fold change±s.d. for WNT3a over vehicle calculated from three independent experiments. (F) Western blotting analysis of ATF4 expression following the indicated shRNA treatments. β-actin is shown as a loading control. (G–J) Effect of *Atf4* knockdown on WNT-induced *Slc1a5* expression (G), glutamine uptake (H,I) or *Slc7a7* expression (I), compared with the effect of control shRNA treatment (shLacZ, shRFP). Data are presented as mean±s.d from three independent experiments. **P*≤0.05, ***P*≤0.005, ****P*≤0.0005, *****P*≤0.00005 [one-way ANOVA with Tukey's post hoc test (A,B,G,H) or unpaired two-tailed Student's *t*-test (C–F,I,J)].

presence of rapamycin (Fig. 4B,C). This is likely due to normal induction of *Slc7a7*, because rapamycin treatment did not affect WNT induction of *Slc7a7* despite completely abrogating *Slc1a5* induction by WNT (Fig. 4D,E). These data indicate that mTORC1 activity is required for both glutamine uptake and the normal expression of both ATF4 and ASCT2. We next sought to determine whether ATF4 activity is required for *Slc1a5* induction by WNT. *Atf4* knockdown using shRNA reduced both ATF4 protein expression and *Slc1a5* mRNA expression in ST2 cells (Fig. 4F,G). Moreover, *Atf4* expression was necessary for *Slc1a5* induction by WNT, because *Atf4* knockdown completely prevented the induction of *Slc1a5* by WNT (Fig. 4F,G). Likewise, *Atf4* knockdown significantly reduced basal glutamine uptake (Fig. 4H). Similar to the rapamycin treatment, *Atf4* knockdown did not affect either WNT-induced glutamine uptake or *Slc7a7* induction by WNT (Fig. 4I,J). Collectively, these data indicate that ATF4 is critical for the majority of glutamine uptake by regulating *Slc1a5* mRNA expression downstream of mTORC1.

Glutamine uptake is required for osteoblast differentiation

We next sought to determine the role of these glutamine transporters during WNT-induced osteoblast differentiation. Knockdown of *Slc7a7* using shRNA reduced the induction of both early osteoblast marker genes (exemplified by *Akp2*, also known as *Alpl*) and terminal osteoblast marker genes (exemplified by *Bglap*) and prevented matrix mineralization in response to WNT (Fig. 5A–D). Interestingly, *Slc1a5* knockdown did not affect early osteoblast marker gene induction (e.g. *Akp2*) but specifically reduced the induction of terminal osteoblast marker genes (e.g. *Bglap*) and prevented matrix mineralization in response to WNT (Fig. 5E–H). These data indicate that both *Slc7a7* and *Slc1a5* are required for WNT-induced osteoblast differentiation in ST2 cells.

DISCUSSION

Increased matrix synthesis associated with bone formation increases the demand for amino acids. It stands to reason that osteoblasts must increase amino acid production or acquisition to meet this biosynthetic demand. Here, we describe the intricate regulation of glutamine acquisition by WNT signaling in an osteoblast progenitor cell line. We identified genes encoding two amino acid transporters, *Slc1a5* and *Slc7a7*, responsible for the majority of glutamine uptake in ST2 cells undergoing osteoblast differentiation in response to WNT. *Slc1a5* encodes the primary glutamine transporter in ST2 cells whereas *Slc7a7* mediates WNT-stimulated glutamine uptake associated with osteoblast differentiation. These transporters appear to work in concert to provide sufficient glutamine, and likely other amino acids, to initiate and sustain osteoblast differentiation and matrix production *in vitro*.

Osteoblast differentiation is characterized by rapid proliferation of osteoblast progenitors followed by differentiation into mature matrix-producing osteoblasts. This process is associated with increased glutamine consumption and metabolism (Fig. 1) (Karner et al., 2015; Yu et al., 2019). Blocking glutamine consumption (Fig. 5) or metabolism prevents osteoblast differentiation and matrix formation (Brown et al., 2011; Karner et al., 2015; Yu et al., 2019). It is not clear why osteoblasts have such an acute requirement for glutamine to facilitate these processes. Osteoblast differentiation is associated with altered energetic and biosynthetic demands (Guntur et al., 2014; Karner and Long, 2017; Riddle and Clemens, 2017). For example, proliferating cells require

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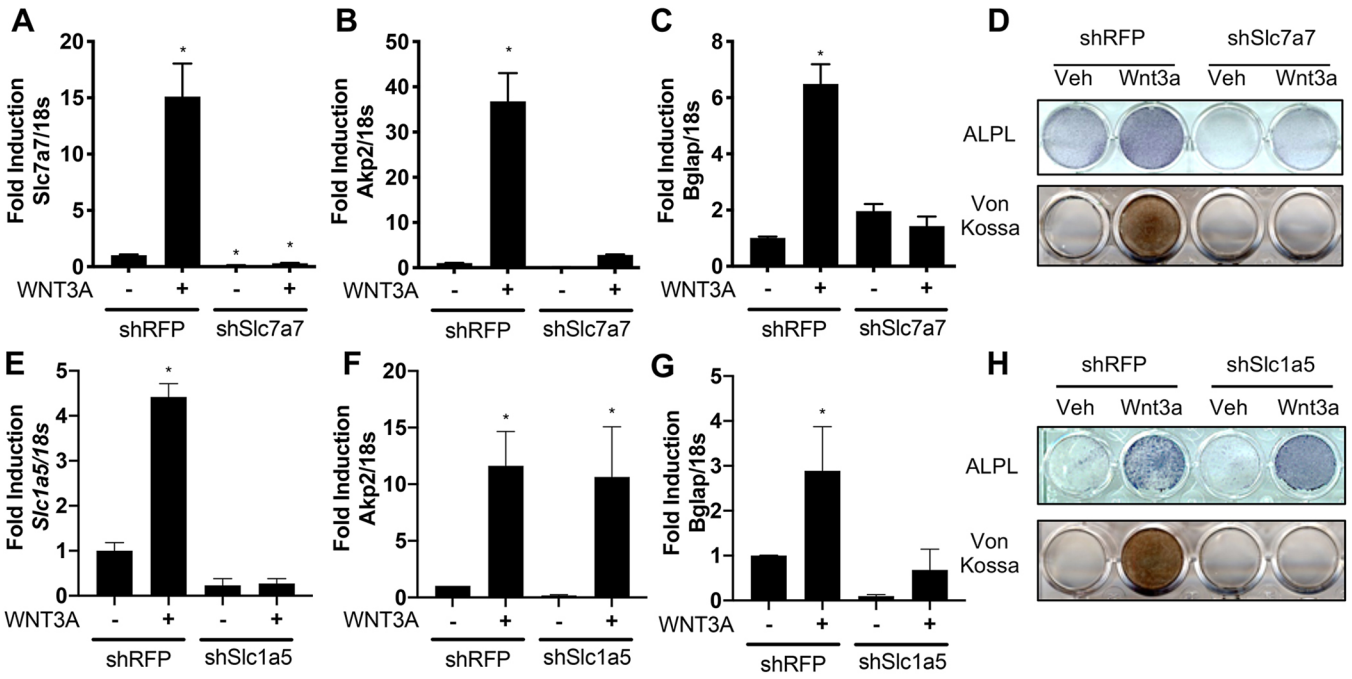

Fig. 5. *Slc7a7* and *Slc1a5* are both required for WNT-induced osteoblast differentiation. Effect of *Slc7a7* (A–D) or *Slc1a5* knockdown (E–H) on WNT-induced gene expression (A–C,E–G), or alkaline phosphatase (ALPL) and von Kossa staining (D,H), compared with the effects of control shRNA treatment (shRFP). Data are presented as mean fold change±s.d. for three independent experiments. *$P \leq 0.05$ (one-way ANOVA with Tukey's post hoc test).

nucleotides and must duplicate their cell mass in order to divide (Hosios et al., 2016). Similarly, differentiation and matrix production is associated with increased protein synthesis and secretion, which may increase reactive oxygen species and oxidative stress detrimental to osteoblast differentiation (Almeida et al., 2007; Mody et al., 2001). Glutamine is a multifunctional amino acid uniquely suited to fulfill these anabolic demands. For example, glutamine-derived nitrogen is important for the *de novo* synthesis of both nucleotides and amino acids. Similarly, glutamine-derived carbon is used for the synthesis of both amino acids and the antioxidant glutathione. Finally, glutamine-derived α-ketoglutarate can provide energy to fulfill energetic demands through entry into the TCA cycle (Newsholme et al., 2003). Thus, osteoblasts likely utilize glutamine disparately during differentiation to fulfill distinct metabolic purposes.

WNT is a potent regulator of osteoblast differentiation and bone formation (Cui et al., 2011; Hill et al., 2005). One of the earliest events associated with WNT-induced osteoblast differentiation is increased glutamine consumption (Fig. 1; Karner et al., 2015). It is interesting to note that glutamine uptake is not only rapidly increased but also sustained throughout the differentiation process. Here, we identified two glutamine transporters encoded by *Slc1a5* and *Slc7a7* that mediate glutamine uptake in ST2 cells. *Slc1a5* is critical for the majority of glutamine uptake (Fig. 2). Conversely, *Slc7a7* is responsible for mediating the acute increase in glutamine uptake in response to WNT. Importantly, the transcription of these transporters is regulated disparately by WNT. First, expression of *Slc7a7* is upregulated within 6 h, whereas *Slc1a5* expression is not increased until 72 h after WNT stimulation. Mechanistically, β-catenin is both necessary and sufficient for the rapid induction of *Slc7a7* expression and the acute increase in glutamine uptake in response to WNT. Conversely, β-catenin is dispensable for *Slc1a5* expression and basal glutamine consumption in ST2 cells (Fig. 3 and data not shown). Rather, *Slc1a5* expression is regulated downstream of mTORC1 and ATF4 in ST2 cells (Fig. 4). We previously determined that WNT induces a GCN2-dependent integrated stress response (ISR) through mTORC1 (Karner et al., 2015). We show here that the ISR transcriptional effector ATF4 is critical for both basal and WNT-induced *Slc1a5* expression and glutamine uptake. This is consistent with recent data demonstrating that *Slc1a5* is directly regulated by ATF4 (Han et al., 2013; Hu et al., 2020).

Collectively, these data support a biphasic model in which WNT signaling regulates glutamine consumption via canonical and noncanonical pathways to facilitate osteoblast differentiation. Interestingly, inhibiting *Slc7a7*, but not *Slc1a5*, inhibited *Akp2* induction, whereas inhibition of either transporter inhibited terminal osteoblast differentiation and matrix mineralization. The precise mechanism underlying this discrepancy is not clear; however, it is important to note that in addition to glutamine, ASCT2 can transport alanine, serine and asparagine. Conversely, γ(+)-Lat1 mediates the influx of ornithine, arginine and lysine in exchange for the efflux of cationic amino acids (Chillaron et al., 1996; Pfeiffer et al., 1999; Torrents et al., 1999), which may be contributing to the disparate early osteoblast differentiation phenotypes observed. It will be important to elucidate the substrates of these amino acid transporters in osteoblast progenitors and determine the precise role they play during WNT-induced osteoblast differentiation.

MATERIALS AND METHODS

Cell culture

The bone marrow-derived cell line, ST2 (RRID: CVCL_2205) was plated at 40,000 cells/ml in α-MEM (GIBCO) supplemented with 10% FBS (Invitrogen). In WNT treatment experiments, 25 ng/ml WNT3a (TIME Bioscience) or vehicle control (0.1% BSA in PBS) was supplemented in the α-MEM. For LiCl treatment, 20 mM LiCl or vehicle control (20 mM NaCl) was added in the growth medium. When inhibitors were used, cells were pretreated with corresponding inhibitors for 30 min before any other treatment. In indicated experiments, growth medium was supplemented with 100 nM rapamycin (Sigma-Aldrich), 250 ng/ml DKK1 (R&D) or

0.03 mM GPNA (MP Biomedicals), or the respective vehicle (DMSO, 0.1% BSA in PBS or 1 M HCl for Rapamycin, DKK1 and GPNA, respectively) as a control. For WNT3a-induced mineralization, cells were treated with WNT3a for 72 h followed by osteogenic medium [α-MEM supplemented with 50 mg/ml ascorbic acid (Sigma-Aldrich) and 10 mM β-glycerophosphate (Sigma-Aldrich)] for 6 days. Osteoblast differentiation was assayed by visualizing alkaline phosphatase activity using 5-bromo-4-chloro-3′-indolyl-phosphate/Nitro Blue tetrazolium (BCIP/NPT; Katagiri et al., 1994) or von Kossa staining of deposited calcium phosphate (Rungby et al., 1993). The alkaline phosphatase assay was performed 24 h after WNT stimulation. Von Kossa staining was performed 6 days after addition of osteogenic medium.

Glutamine uptake assay

Cells were washed with PBS and two additional washes with Krebs Ringer Hepes (KRH; 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM $NaHCO_3$, 5 mM HEPES, pH 8 and 1 mM D-glucose). Cells were then treated for 5 min with KRH containing 4 µCi/ml L-(2,3,4-^{3}H)-glutamine. Glutamine uptake and cellular metabolism was terminated by washing with ice-cold KRH and scraping cells with 1 ml ice-cold dH_2O. The lysate was centrifuged, and counts per minute (CPM) were measured using a Beckman LS6500 scintillation counter. All results are normalized to cell number. In experiments to measure glutamine transport system activity, the recipe for KRH was altered as follows: complete KRH (120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.4 mM KH_2PO_4, 5 mM HEPES, 1 mM D-glucose, pH 8), complete KRH plus MeAIB (120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.4 mM KH_2PO_4, 5 mM HEPES, 5 mM MeAIB, 1 mM D-glucose, pH 8), complete KRH plus GPNA (120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.4 mM KH_2PO_4, 5 mM HEPES, 0.3 mM GPNA, 1 mM D-glucose, pH 8), Na^+-free KRH (120 mM choline chloride, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.4 mM KH_2PO_4, 5 mM HEPES, 1 mM D-glucose, pH 8), Na^+-free plus Li^+ KRH (120 mM LiCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.4 mM KH_2PO_4, 5 mM HEPES, 1 mM D-glucose, pH 8). We calculated the relative glutamine transport system activity as follows:

$$\%\ System\ A\ activity = \frac{CPM_{\text{complete KRH}} - CPM_{\text{MeAIB}}}{CPM_{\text{complete KRH}}} \times 100$$

$$\%\ System\ N\ activity = \frac{(CPM_{Li^+}) - (CPM_{Na^+\text{free}})}{CPM_{\text{complete KRH}}} \times 100$$

$$\%\ System\ L\ activity = \frac{CPM_{Na^+\text{free}}}{CPM_{\text{complete KRH}}} \times 100$$

$$\%\ System\ ASC\ and\ \gamma(+)L\ activity = 100 - \%\ System\ N\ activity - \%\ System\ L\ activity.$$

shRNA knockdowns

Lentiviral vectors were obtained from the shRNA consortium at Washington University School of Medicine. All knockdown results were confirmed using two or more unrelated shRNA constructs. The shRNA sequence listed first was shown in the results. The lentiviral vector pLKOpuro (RRID: Addgene_8453) was modified to express shRNAs targeting *Slc1a5* (5′-CCTGTAGAGTTCTCTACCCTT-3′, 5′-GCAGTGTTCATCGCACA-ACTA-3′), *Slc7a7* (5′-GCTACATGTTTCAGACTTCAT-3′, 5′-GCCATC-TGTATGGTTCATG-3′), *Atf4* (5′-CCAGAGCATTCCTTTAGTTTA-3′, 5′-CCTCTAGTCCAAGAGACTAAT-3′), *Catnnb1* (5′-GCGTTATCAA-ACCCTAGCCTT-3′, 5′-CCATCACAGATGTTGAAACAT-3′), or either RFP (5′-ACAACAGCCACAACGTCTATA-3′) or LacZ (5′-GCGATCGT-AATCACCCGAGTG-3′) as a negative control. The shRNA-expressing lentiviral vector was co-transfected in 293T cells with the plasmids pMD2.g (RRID: Addgene_12259) and psPax2 (RRID: Addgene_12260). Virus-containing medium was collected and filtered. ST2 cells were infected for 24 h and recovered for 24 h in regular medium prior to further treatment.

RNA isolation and RT-qPCR

Total RNA was isolated using an RNAeasy kit with on-column DNase treatment (Qiagen). 500 ng of total RNA was reverse transcribed using the Iscript cDNA Synthesis kit (Bio-Rad). Reactions were set up in technical and biological triplicate in a 96-well format on an ABI Quantstudio 3 using SYBR Green chemistry (SsoAdvanced; Bio-Rad). The PCR conditions were 95°C for 3 min followed by 35 cycles of 95°C for 10 s and 60°C for 30 s. Gene expression was normalized to 18S rRNA, and relative expression was calculated using the $2^{-(\Delta\Delta Ct)}$ method. Primers were used at 0.1 µM, and their sequences are listed in Table S1. PCR efficiency was optimized, and melting curve analyses of products were performed to ensure reaction specificity.

Western blotting

ST2 cells were scraped in lysis buffer containing 50 mM Tris (pH 7.4), 15 mM NaCl, 0.5% NP-40, 0.1% SDS and 0.1% sodium deoxycholate with a protease and phosphatase inhibitor tablet (Roche). Protein concentration was quantified using the BCA method (Pierce). Proteins (20 µg) were resolved on 12% polyacrylamide gels and transferred onto Immuno-Blot PVDF membrane (Bio-Rad). Proteins were detected using the following specific antibodies: anti-ASCT2 (RRID: AB_10891440), anti-Slc7a7 (RRID: AB_2576546), anti-α-tubulin (RRID: AB_2619646), anti-ATF4 (RRID: AB_2058752), anti-β-actin (RRID: AB_330288), anti-phospho-S6 (Ser240/244) (RRID: AB_10694233), anti-S6 (RRID: AB_331355) and anti-β-catenin (RRID: AB_634603). Further antibody information is provided in Table S2. The membranes were blocked for 1 h at room temperature in 5% milk powder in TBS with 0.1% Tween (TBST) and then incubated at 4°C with the primary antibody overnight. Membranes were washed three times with TBST and further incubated with anti-rabbit IgG, HRP-linked antibody (RRID: AB_2099233) in 5% milk (in TBST) for 1 h at room temperature. All blots were developed using enhanced chemiluminescence (Clarity Substrate Kit, Bio-Rad). Each experiment was repeated with a minimum of three independently prepared protein samples.

Statistics

Statistical significance was determined by one-way ANOVA or by an unpaired two-tailed Student's *t*-test.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.M.K.; Methodology: L.S., D.S.; Validation: L.S., D.S.; Formal analysis: L.S., D.S.; Investigation: L.S., D.S., Y.Y., C.M.K.; Resources: F.L., C.M.K.; Data curation: L.S., C.M.K.; Writing - original draft: L.S., D.S., C.M.K.; Writing - review & editing: L.S., D.S., C.M.K.; Visualization: L.S., D.S., C.M.K.; Supervision: C.M.K.; Project administration: C.M.K.; Funding acquisition: F.L., C.M.K.

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