Gene Regulation and Systems Biology

ORIGINAL RESEARCH

Yin Yang 1 and Adipogenic Gene Network Expression in Longissimus Muscle of Beef Cattle in Response to Nutritional Management

Sonia J. Moisá[1,2], Daniel W. Shike[2], William T. Meteer[2], Duane Keisler[3], Dan B. Faulkner[2] and Juan J. Loor[1,2,4]

[1]Mammalian NutriPhysioGenomics, University of Illinois, Urbana, USA. [2]Department of Animal Sciences, University of Illinois, Urbana, USA. [3]Department of Animal Sciences, University of Missouri, Columbia, USA. [4]Division of Nutritional Sciences, University of Illinois, Urbana, USA. Corresponding author email: jloor@illinois.edu

Abstract: Among 36 differentially-expressed genes during growth in longissimus muscle (LM) of Angus steers, Yin Yang 1 (YY1) had the most relationships with other genes including some associated with adipocyte differentiation. The objective of this study was to examine the effect of nutritional management on mRNA expression of YY1 along with its targets genes *PPARG*, *GTF2B*, *KAT2B*, *IGFBP5* and *STAT5B*. Longissimus from Angus and Angus × Simmental steers (7 total/treatment) on early weaning plus high-starch (EWS), normal weaning plus starch creep feeding (NWS), or normal weaning without starch creep feeding (NWN) was biopsied at 0, 96, and 240 days on treatments. Results suggest that YY1 does not exert control of adipogenesis in LM, and its expression is not sensitive to weaning age. Among the YY1-related genes, EWS led to greater *IGFBP5* during growing and finishing phases. Pro-adipogenic transcriptional regulation was detected in EWS due to greater *PPARG* and *VDR* at 96 and 240 d vs. 0 d. *GTF2B* and *KAT2B* expression was lower in response to NWS and EWS than NWN, and was most pronounced at 240 d. The increase in *PPARG* and *GTF2B* expression between 96 and 240 d underscored the existence of a molecular programming mechanism that was sensitive to age and dietary starch. Such response partly explains the greater carcass fat deposition observed in response to NWS.

Keywords: early weaning, gene expression, adipogenesis, nutrition, Yin Yang 1, steers

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Background

Recent transcriptomics and bioinformatics research by our group revealed that, among 36 transcription regulators differentially-expressed during growth in longissimus muscle (LM) of early-weaned Angus steers, Yin Yang 1 (YY1) had the most relationships with other differentially expressed genes (with a total of 5,616).[1] In non-ruminants, YY1 is a ubiquitously-expressed DNA-binding zinc-finger transcription factor implicated in the regulation of genes that are important for cell growth, development, differentiation, cell cycle, and programmed cell death.[2]

Expression of YY1 in bovine tissue appears to be greater in fat cells of developing tissue as demonstrated by data from mammary fat pad vs. mammary epithelium.[3] Because the differentiation of 3T3-L1 preadipocytes to adipocytes is dependent on the suppression of the expression of *CHOP*-10 (a member of the C/EBP family) by YY1,[4] this transcription factor also could play a role in intramuscular fat (IMF) deposition of young beef cattle. Bioinformatics analysis using Ingenuity Pathway Analysis® (IPA; Ingenuity systems, CA, USA) revealed that YY1 interacts with insulin-like growth factor binding protein 5 (*IGFBP*5), signal transducer and activator of transcription 5B (*STAT*5*B*), general transcription factor 2B (*GTF*2*B*), and K(lysine) acetyltransferase 2B (*KAT*2*B*). The latter two play a role in chromatin remodeling (SWItch/Sucrose Non-Fermentable (SWI/SNF) and CBP/p300) and gene transcription.[5]

Previous work has demonstrated that weaning beef calves earlier than the common 205 days of age and feeding high dietary starch, compared with high dietary fiber, leads to marked upregulation of adipogenic and lipogenic genes in *LM*.[6] Such nutritional management strategy often enhances carcass fat deposition.[7,8] However, it remains unknown if expression of YY1 and its known interacting partners is responsive to nutritional management or whether it is associated with carcass fat deposition.

The general hypothesis of the present study was that longitudinal expression of YY1, its closely-related genes, and *PPARG* within LM would be altered by nutritional management in LM of growing steers. As an initial step in establishing a role for YY1 in LM, the specific objective was to profile gene expression of LM tissue from steers managed to consume different levels of dietary energy at an earlier or normal weaning age. Those data were combined with concentrations of hormones in blood as a way to better understand the system in a more holistic fashion.

Methods

Animal management

All experimental procedures involving steers were approved by the University of Illinois Institutional Animal Care and Use Committee under protocol no. 09143. Angus (A) and Angus × Simmental (SA) steer calves from the University of Illinois beef cattle herd were utilized. Prior to the start of the study, 12 A and 9 SA steers were randomly assigned to early weaning (129 ± 36 days of age) plus a high-starch diet (EWS, 4 A and 3 SA), normal weaning (225 ± 36 days of age) plus corn-based creep supplement (NWS, 4 A and 3 SA), and normal weaning with no creep feeding (NWN, 4 A and 3 S A). The latter reflects closely the current trends in management in the US industry. Steers in NWS had access to creep feeders in the pasture at time of early weaning. The EWS steers were fed ad libitum during their growing phase and, when NWS and NWN steers joined the feedlot with the EWS, all steers received the same finishing diet ad libitum. Table 1 reports the composition of the diet and creep supplement. The vitamin fraction of the dietary supplement contained 680,329 IU vitamin A/kg of dry matter, 68,039 IU vitamin D_3/kg of dry matter, and 9,072 IU vitamin E/kg of dry matter.

Table 1. Composition of the early wean high-starch diet (EWS), the corn-grain-based creep supplement fed to normal-wean steers (NWS), and the finishing diet offered to all steers the day after until harvest (finishing phase).

Ingredient (g/kg dry matter)	EWS	NWS	Finishing diet
Corn husklage	–	–	642
Ground hay	162	–	–
Dry whole kernel corn	668	820	–
Dry rolled corn	–	–	101
Wet corn gluten feed	–	–	210
Supplement[a]	170	179	–
Supplement[b]	–	–	46

Notes: [a]Composition of growing phase supplement (g/kg DM): Soybean Meal (90.2), Limestone (90), Trace Mineralized Salt (6.7), Copper Sulfate (0.03), Vitamin ADE (0.7); [b]composition of finishing phase supplement (g/kg DM): Ground Corn (844), Limestone (110), Urea (25), Trace Mineralized Salt (1), Thiamine (2.5), Copper Sulfate (0.5) and Vitamin ADE (1.0).

For the 96 d treatment period (ie, growing phase) EWS calves remained in the feedlot and NWS and NWN dams nursed their calves and grazed mixed pastures of endophyte-infected tall fescue (*Festuca arundinacea*), orchardgrass (*Dactylis glomerata*), bluegrass (*Poa pratensis*), red clover (*Trifolium pratense*), white clover (*Trifolium repens*), and alfalfa (*Medicago sativa*). Calves in NWS and NWN were weaned at 225 days of age and joined the early-weaned calves at the feedlot at which point both groups were fed a common finishing diet until slaughter (Table 1). Seven animals within EWS (n = 4 A, 3 SA), NWS (n = 4 A, 3 SA), and NWN (n = 4 A, 3 SA) were selected for biopsies. LM muscle biopsies were collected via biopsy from the same steers at 0 (early weaning), 96 (normal weaning), and 240 days after early weaning for transcript profiling using quantitative RT-PCR. 10 mL of blood was collected from the jugular vein before biopsies to isolate serum for metabolites by radioimmunoassay, following standard protocols at the Veterinary Diagnostics Laboratory, College of Veterinary Medicine, University of Illinois, Urbana-Champaign. Serum was obtained by centrifugation at 2,000× g for 15 min at 25 °C. Growth hormone (GH), insulin-like growth factor-1 (IGF-1), and leptin were measured via RIA as described previously.[9,10]

Yin Yang 1 gene network expression

An investigation using the 126 genes linked to YY1 in LM from our previous microarray study[1] was used to assess which genes could be related to a biological process associated with myogenesis and adipogenesis. The relative level of expression of YY1 and its target genes in adipose tissue was the first parameter that we had to corroborate during the literature search; once the expression level of a gene and the possible role in adipose tissue was confirmed, it was chosen for qPCR in LM. The selection based on literature searches resulted in the identification of *IGFBP5*, *STAT5B*, *GTF2B*, and *KAT2B*. These 4 genes act as links between YY1 and RXR β (*RXRB*), *PPARG*, and *VDR*.

Primer design and evaluation

Primers were designed using Primer Express 3.0 with minimum amplicon size of 100 bp and limited 3′ G+C (Applied Biosystems, CA). When possible, primers were designed to fall across exon–exon junctions. Primers were aligned against publicly available databases using BLASTN at NCBI and UCSC's Bos taurus Genome Browser Gateway. Prior to qPCR, primers were tested in a 20 µL PCR reaction using the same protocol described for qPCR except for the final dissociation protocol. For primer testing we used a universal reference cDNA (RNA mixture from 5 different bovine tissues) to ensure identification of desired genes. Five µL of the PCR product were run in a 2% agarose gel stained with ethidium bromide (2 µL). The remaining 15 µL were cleaned using QIAquick® PCR Purification Kit (QIAGEN) and sequenced at the Core DNA Sequencing Facility of the Roy J. Carver Biotechnology Center at the University of Illinois, Urbana-Champaign. Only those primers that did not present primer-dimer, had a single band at the expected size in the gel, and had the right amplification product (verified by sequencing) were used for qPCR. The accuracy of a primer pair also was evaluated by the presence of a unique peak during the dissociation step at the end of qPCR. Additional information is available in Tables 2 and 3.

Biopsy, RNA extraction and PCR

Details of all these procedures have been reported previously.[6] Briefly, a needle biopsy gun fitted with a 12 gauge biopsy needle was used to harvest ~0.5 g of tissue (Bard Magnum, C. R. Bard, Covington, GA, USA). Trizol extraction of RNA was exactly as reported previously.[11] qPCR data were calculated with the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems, CA). The final data were normalized using the geometric mean of the genes *UXT*, *MTG1* and *RPS15A*, which were previously identified as suitable internal control genes in LM 11. Additional details of qPCR performance are in the Table 4.

Statistical analysis

Quantitative PCR data were analyzed using the MIXED procedure of SAS (SAS 9.1 Institute, Cary, NC, USA). Before statistical analysis, normalized qPCR data were transformed to fold-change relative to day 0 (ie, early weaning day). To estimate standard errors at day 0 and prevent biases in statistical analysis, normalized qPCR data were transformed to

Table 2. Gene ID, GenBank accession number, sequence and amplicon size of primers used to analyze gene expression by qPCR.

Gene ID	Accession #	Gene	Primers[a]	Primers (5′-3′)	bp[b]
513231	NM_001046142.1	*GTF2B*	F.807	CTGGAAGGAGCCCGATATCTG	100
		GTF2B	R.906	CCAGCAATATCTCCAATCTCTTTTT	
404185	NM_001105327.1	*IGFBP5*	F.220	GAGCAAGCCAAGATCGAAAG	90
		IGFBP5	R.309	GAAGATCTTGGGCGAGTAGG	
407215	XM_002684716.1	*KAT2B*	F.1723	CAAGTCAAGGGCTATGGAACACA	100
		KAT2B	R.1822	TTGCGTATTCATCTGCGTAGGT	
281993	BC116098.1	*PPARG*	F.1356	GAGCCCAAGTTCGAGTTTGC	100
		PPARG	R.1455	GGCGGTCTCCACTGAGAATAAT	
504943	NM_001083640.1	*RXRB*	F.1318	CTGCGGGCAATCATTCTGTT	100
		RXRB	R.1417	AGGTCTCCAGGGATGCATATACTT	
282376	NM_174617.3	*STAT5B*	F.130	TCATCAGATGCAAGCGCTGTA	105
		STAT5B	R.234	TTATCAAGATCTATTGAGTCCCAAGCT	
533656	NM_001167932.1	*VDR*	F.933	GTCATCATGCTTCGCTCCAA	101
		VDR	R.1033	CTGGTCACGTCGCTGACTTG	
534353	NM_001098081.1	YY1	F.1120	ACGACACCAACTGGTCCATACTG	100
		YY1	R.1219	CACATGTGTGCGCAAATTGA	

Notes: [a]Primer direction (F—forward; R—reverse) and hybridization position on the sequence; [b]amplicon size in base pairs (bp).

obtain a perfect mean of 1.0 at day 0, leaving the proportional difference between the biological replicate. The same proportional change was calculated at all other time points to obtain a fold-change relative to day 0. Fixed effects in the statistical model for each gene and blood parameters analyzed included treatment, breed, time, treatment × breed, treatment × time, and breed × treatment × time. Gene and blood data analysis of the three time points studied included a repeated-measures statement with an autoregressive covariate structure. Age at weaning was used as a covariate in the model. The random effect in all models was steer within treatment. The statistical model used was:

$$Y_{ijklm} = \mu + C_i + T_j + B_k + S_l + b_1 (A_{ijklm} - \bar{A}) + (C \times T)_{ij} + (T \times B)_{jk} + (C \times B)_{ik} + (T \times C \times B)_{ijk} + \varepsilon_{ijklm}$$

where, Y_{ijklm} is the background-adjusted normalized fold change or blood data value; μ is the overall mean; C_i is the fixed effect of time (3 levels); T_j is the fixed effect of treatment (2 levels); B_k is the fixed effect of breed (2 levels); S_l is the random effect of

Table 3. Sequencing results of PCR products from primers of genes designed for this experiment. Best hits using BLASTN (http://www.ncbi.nlm.nih.gov) are shown.

Gene	Sequence
GTF2B	GGTTGTCTTTTTGGCCAGGGGCATCAGGCCTGAGAAAAAGGACCCAAAAGAGATTTGGAGATATTTGC TGGGATCCCTTTTCCCC
IGFBP5	ATTCGCCCAGCGTGCATCTGCTGGTGCGTGGACAAGGTACGGGATGAAGCTGCCGGGCATGG AGTACGTGGACGGGGACTTTCAGTGCCACACCTTCGAGA
KAT2B	GGAAAACTTGAGATCTGGGTTTTTAGTGTGATATTCCTTCAAGGTGTTCATCAGGTGTGTTCCATA GCCCTTGACTTGGAA
PPARG	TAACAGTACTCTCCTAAAATACGGCGTGCACGAGATCATTTACACGATGCTGGCCTCCTTGATGAA
RXRB	CGACGCGGTCAGCGTCGTCGTCGTCTTCCGTCTCTCAGGGTCCATAGGCCTTAAGTGTCTAGAG CATCTGTTTTTCTTCACA
STAT5B	GCCATCATGTAGGGTCGCCATTACTTATCCCAGGTGATTGAAGGGCCAAGCTTGGGGACTCAATAGAT CTTGATAAAAAACCCC
VDR	GTCGCGTCCGGGTCCCGGCACATGTCGTCAGGGGGTAAGGACTGGTTGGAGCCGAAGCCATGATGA CCTTCCAATGGCAACC
YY1	GGACCTTCGGTCCGCTTCAAGGCTGCGGGGAAACGCTTTTCACTGGACTTCAATTTGCGCACACATGTGA

Table 4. qPCR Performance.

Gene	Median Ct[a]	Median ΔCt[b]	Slope[c]	(R^2)[d]	Efficiency[e]
GTF2B	28.536	3.937	–2.961	0.993	2.17
IGFBP5	23.035	–1.497	–3.407	0.997	1.96
KAT2B	23.331	–1.140	–3.564	0.995	1.90
MTG1	26.363	–	–3.490	0.990	1.93
PPARG	27.552	2.790	–3.465	0.991	1.94
RPS15A	22.227	–	–3.165	0.991	2.07
RXRB	26.538	1.986	–3.147	0.990	2.07
STAT5B	22.358	–2.372	–3.345	0.998	1.99
VDR	29.511	4.860	–3.192	0.986	2.05
UXT	25.892	–	–3.470	0.992	1.94
YY1	25.703	1.024	–3.017	0.995	2.14

Notes: [a]Calculated considering all time points and all animals; [b]calculated as [Ct gene—geometrical mean of Ct internal controls] for each time point and each animal; [c]slope of the standard curve; [d]R^2 = coefficient of determination of the standard curve; [e]efficiency is calculated as [$10^{(-1/Slope)}$].

steer nested within treatment; b_1 is the regression coefficient for the covariate A, where A was the age of the animal at weaning; C × T, T × B, C × B are the interactions of time by treatment, treatment by breed and time by breed, respectively; T × C × B is the interaction or third order for the main effects; and ε_{ijklm} is the random error $(0, \sigma_e^2)$ associated with Y_{ijklm}. A likelihood ratio test was used in order to examine if the main effects were non-significant, and if they could have an impact on the logarithm of convergence of the original model. Breed effect had no significant impact on the logarithm of convergence of the model, thus, breed remained in the model.

Moreover, partial Pearson correlation analysis among genes, adjusted for the fixed effects, was conducted using PROC CORR in SAS (Table 6).

Results

Blood metabolites

The concentration of GH was lower (interaction $P < 0.05$) in both EWS and NWS than NWN at 96 d (Table 5). However, IGF-1 concentrations were lower in EWS than in NWS and NWN steers at 96 days. Leptin concentrations were greater (interaction $P < 0.06$) in NWS than EWS and NWN at 96 d. At 240 d, concentrations of GH and leptin in EWS steers were intermediate to those in NWN ($P < 0.05$) and NWS ($P < 0.05$). However, EWS steers had lower IGF-1 at 240 d than in NWN and NWS. For these three metabolites there was no significant breed effect.

Carcass traits

There was no difference in HCW between EWS, NWS, and NWN steers. Even though carcass marbling score did not have a significant treatment × breed interaction ($P = 0.41$; Table 6), it had a significant treatment effect ($P = 0.01$), with greater values in EWS steers compared with NWS and NWN steers. This result led to a greater percentage of steers grading Choice or greater (data not shown). Back fat had a treatment × breed interaction ($P = 0.02$) with a breed

Table 5. Serum concentration (ng/mL) of leptin, growth hormone (GH), and IGF-1 of steers (n = 7/treatment) managed under three nutritional treatments during the growing phase (0 to 96 d) followed by feeding a common high-starch diet during the finishing phase (97 to 240 d).

Item	Treatments*			SEM#	*P* value¶
	EWS	NWS	NWN		
GH					
0 d	14.6	13.0	17.6	2.3	0.001
96 d	8.3[b]	4.1[b]	15.7[a]		
240 d	18.4[b]	6.3[c]	25.3[a]		
IGF-1					
0 d	77[b]	128[a]	142[a]	11	0.06
96 d	132[b]	193[a]	172[a]		
240 d	164[b]	229[a]	234[a]		
Leptin					
0 d	3.9	1.9	3.7	1.3	0.001
96 d	3.2[b]	6.7[a]	3.2[b]		
240 d	9.3[a,b]	10.6[a]	6.4[b]		

Notes: *EWS = early weaning plus a high-starch diet; NWS = normal weaning plus corn-grain supplement; NWN = normal weaning without supplement; #standard error of the mean; ¶treatment × time effect; [a–c]Different superscripts across treatments at a given time point for each item denote differences between treatments.

Table 6. Carcass quality parameters of Angus (A) and Angus × Simmental (SA) steers (n = 7/treatment) managed under three nutritional treatments during the growing phase (0 to 96 d) followed by feeding a common high-starch diet during the finishing phase (97 to 240 d).

Item	Treatments[a]						SEM[b]	*P* value[c]		
	EWS		NWS		NWN			Trt	Breed	T × B
	A	SA	A	SA	A	SA				
n	4	3	4	3	4	3				
Hot carcass weight (kg)	294	331	339	345	323	353	19	0.13	0.06	0.53
Marbling score[d]	563	567	520	408	412	390	58	0.01	0.26	0.41
Back fat (mm)	13	10	14	12	11	13	11	0.22	0.06	0.02
REA (mm)	720	750	730	780	770	830	50	0.26	0.15	0.93

Notes: [a]EWS = early weaning plus a high-starch diet; NWS = normal weaning plus corn-grain supplement; NWN = normal weaning without supplement; [b]standard error of the mean; [c]Trt = effect of nutritional management; Breed = B; T × B = interaction of nutritional management and breed; [d]marbling score: 400–499—Small; 500–599—Modest; 600–699—Moderate. REA = ribeye area.

effect ($P = 0.06$) due to greater values with A steers in EWS and NWS, but greater values with SA steers in NWN.

Gene expression

Results for YY1 indicated that there was no time × treatment effect ($P = 0.34$) but there was an overall effect of time ($P < 0.05$) on mRNA expression of all genes, ie, expression of YY1 increased throughout the study with highest expression at 240 d (Fig. 1). Among genes directly linked with YY1, there was a treatment × time interaction ($P = 0.06$) for *RXRB* due to greater expression for NWN compared with EWS at 240 days, and a strong negative correlation with YY1 and a positive correlation with *VDR*, *IGFBP5* and *STAT5B* (Table 6).

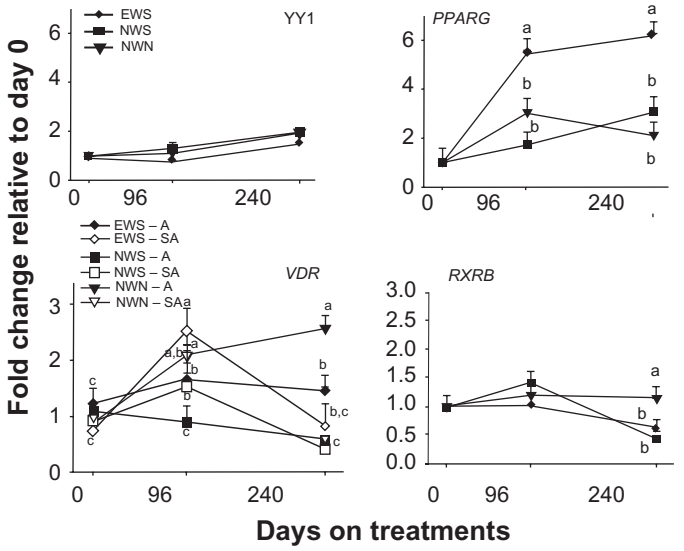

Figure 1. Patterns of mRNA expression of Ying Yang 1 (YY1), peroxisome proliferator-activated receptor γ (*PPARG*), vitamin D receptor (*VDR*), and retinoic X receptor β (*RXRB*).
Notes: Fold-changes in expression are expressed relative to day 0. [a–c]Different superscript letters denote significant treatment × time ($P < 0.05$) or for VDR treatment × time × breed effects ($P < 0.05$).

The *VDR* gene was the only gene with a significant ($P = 0.0006$) breed × treatment × time interaction due to a gradual increase in expression during the growing phase with both EWS and NWN vs. NWS. Expression of *VDR* was lower during the finishing phase for both EWS and NWS but remained unchanged with NWN steers. Overall expression of *VDR* at 240 days was highest for NWN, intermediate with EWS, and lowest with NWS. There was a strong negative correlation between *VDR*, *IGFBP5*, *RXRB*, and *STAT5B* with YY1 in animals in NWS ($P < 0.01$), with *RXRB* in EWS ($P = 0.02$), and with *STAT5B* in NWN ($P = 0.05$); whereas, there was a positive correlation between YY1 with *KAT2B* ($P = 0.001$) and *GTF2B* ($P = 0.001$) in NWN, with *IGFBP5* ($P = 0.01$) and *STAT5B* ($P = 0.004$) in NWS, and with *GTF2B* ($P = 0.001$) and *KAT2B* ($P = 0.05$) in EWS (Table 6).

There was a treatment × time interaction ($P < 0.01$) for the expression of *PPARG* due to a gradual increase between 0 and 96 d at which point expression was highest with EWS compared with NWS and NWN. During the finishing phase, expression of *PPARG* remained higher with EWS and also NWS, while it decreased due to NWN. Thus, at 240 days the expression of *PPARG* was markedly higher for EWS and not different for NWS and NWN ($P < 0.01$). In the EWS group, there was a positive correlation with *IGFBP5* and *KAT2B* (Table 7). However, in both NWS and

Table 7. Pearson correlation coefficients for genes belong to YY1 network adjusted for the fixed effects.

	IGFBP5	*KAT2B*	*PPARG*	*RXRB*	*STAT5B*	*VDR*	*YY1*
Early weaning							
GTF2B							
R	0.185	0.636	0.185	–0.099	0.177	0.033	0.819
P value	0.241	0.001	0.267	0.532	0.263	0.845	0.001
IGFBP5							
R		0.691	0.513	0.501	0.290	0.589	–0.097
P value		0.001	0.001	0.001	0.062	0.001	0.542
KAT2B							
R			0.514	0.287	0.387	0.385	0.307
P value			0.001	0.065	0.011	0.017	0.048
PPARG							
R				0.035	–0.261	0.620	0.111
P value				0.834	0.114	0.001	0.507
RXRB							
R					0.554	0.377	–0.358
P value					0.001	0.02	0.020
STAT5B							
R						0.097	–0.239
P value						0.562	0.128
VDR							
R							–0.240
P value							0.146
Normal weaning—creep feeding							
GTF2B							
R	–0.143	0.514	0.499	–0.077	–0.253	–0.192	0.835
P value	0.3667	0.001	0.001	0.626	0.106	0.223	0.001
IGFBP5							
R		0.170	–0.216	0.664	0.868	0.657	–0.377
P value		0.282	0.169	0.001	0.001	0.001	0.014
KAT2B							
R			0.491	0.304	0.064	0.178	0.293
P value			0.001	0.050	0.687	0.259	0.06
PPARG							
R				–0.038	–0.295	–0.143	0.444
P value				0.812	0.058	0.365	0.003
RXRB							
R					0.565	0.776	–0.339
P value					0.001	0.001	0.028
STAT5B							
R						0.531	–0.401
P value						0.001	0.004
VDR							
R							–0.401
P value							0.001
Normal weaning—no creep feeding							
GTF2B							
R	–0.160	0.692	–0.057	0.072	–0.323	0.164	0.848
P value	0.311	0.001	0.722	0.648	0.037	0.301	0.001
IGFBP5							
R		0.037	0.108	0.194	0.273	0.114	–0.203
P value		0.817	0.497	0.218	0.08	0.473	0.196
KAT2B							
R			0.191	0.186	–0.098	0.360	0.663
P value			0.227	0.238	0.537	0.019	0.001

(*Continued*)

Table 7. (*Continued*).

	IGFBP5	*KAT2B*	*PPARG*	*RXRB*	*STAT5B*	*VDR*	YY1
PPARG							
R				0.036	–0.345	0.541	–0.137
P value				0.820	0.025	0.001	0.386
RXRB							
R					0.062	0.074	0.143
P value					0.698	0.640	0.366
STAT5B							
R						–0.192	–0.306
P value						0.222	0.049
VDR							
R							–0.093
P value							0.557

NWN there was a strong negative correlation between *PPARG* with *STAT5B* and in NWS only, between YY1, *GTF2B* and *KAT2B* (Table 7).

There was a time × treatment interaction ($P < 0.01$) and a breed × treatment interaction ($P = 0.04$) for *IGFBP5*. The former was due mainly to a longitudinal increase in expression with EWS but a decrease with both NWS and NWN leading to greater expression with EWS at 96 and 240 d (Fig. 2). In the NWS group, there was a negative correlation between YY1 and *IGFBP5* and a strong positive correlation between YY1 and *STAT5B*. In the EWS group, there was a positive correlation between *IGFBP5* with *KAT2B* and also between *IGFBP5* and *STAT5B* (Table 7).

In the case of *GTF2B*, there was a time × treatment interaction ($P < 0.01$) due to a gradual increase in expression primarily with NWN compared with EWS and NWS. Both of those treatments had the same response during the growing and the finishing periods resulting in greater expression at 96 and 240 days than at 0 d, however it was never higher than for NWN (Fig. 2). In all treatments there was a marked positive correlation between *GTF2B* with YY1 and between *GTF2B* and *KAT2B*. The correlation between *GTF2B* and *PPARG* was positive in the NWS group. However, a negative correlation was found between *GTF2B* and *STAT5B* in the NWN group (Table 7).

Expression of *KAT2B* had a time × treatment interaction ($P = 0.02$) due to a longitudinal increase in expression that was lower with NWS at 96 d, and with both NWS and EWS than NWN at 240 d. Moreover, *KAT2B* was the only gene from the ones related to YY1 with an effect of age at weaning ($P < 0.01$, data not shown). During the finishing period, all treatments had greater *KAT2B* expression relative to 0 and 96 d, however at 240 d expression was highest with NWN, intermediate with EWS, and lowest with NWS (Fig. 2). In the EWS group, there was a positive correlation between *KAT2B* expression and that of *GTF2B*, *IGFBP5*, *PPARG*, *STAT5B*, *VDR,* and YY1. In the NWS group there was a positive correlation between *KAT2B* expression and that of *GTF2B*, *PPARG,* and *RXRB*. In the NWN group there was a positive correlation between *KAT2B* with *GTF2B*, *VDR*, and

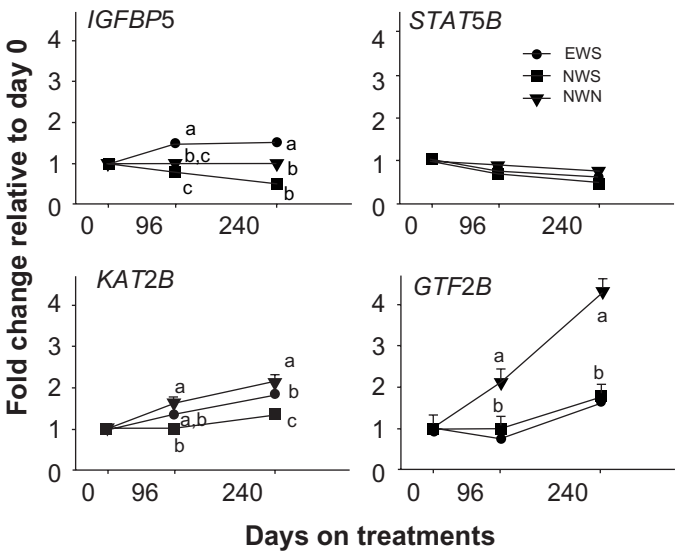

Figure 2. Patterns of mRNA expression of insulin-like growth factor binding protein 5 (*IGFBP5*), signal transducer and activator of transcription 5B (*STAT5B*), K(lysine) acetyltransferase 2B (*KAT2B*), and general transcription factor 2B (*GTF2B*).
Notes: Fold-changes in expression are expressed relative to day 0. [a–c]Different superscript letters denote significant difference ($P < 0.05$) due to treatment × time. There was on overall effect of time ($P < 0.05$) on mRNA expression of all genes.

YY1 (Table 7). Figure 3 depicts a summary of the relationships among genes and the overall response between treatments over time.

Discussion

Nutritional management and carcass traits

Greater marbling scores in EWS steers, leading to a greater percentage of steers grading Choice or greater (data not shown), were likely due to the early feeding of starch.[6] This is expected to enhance ruminal propionate production with a resulting increase in serum insulin and glucose concentrations.[12] As propionate is the major glucogenic volatile fatty acid, more glucose is generated in the liver, resulting in more net energy available to the animal. A pro-insulinemic response would have led to greater glucose uptake by adipose. Despite the similar amount of time required for all

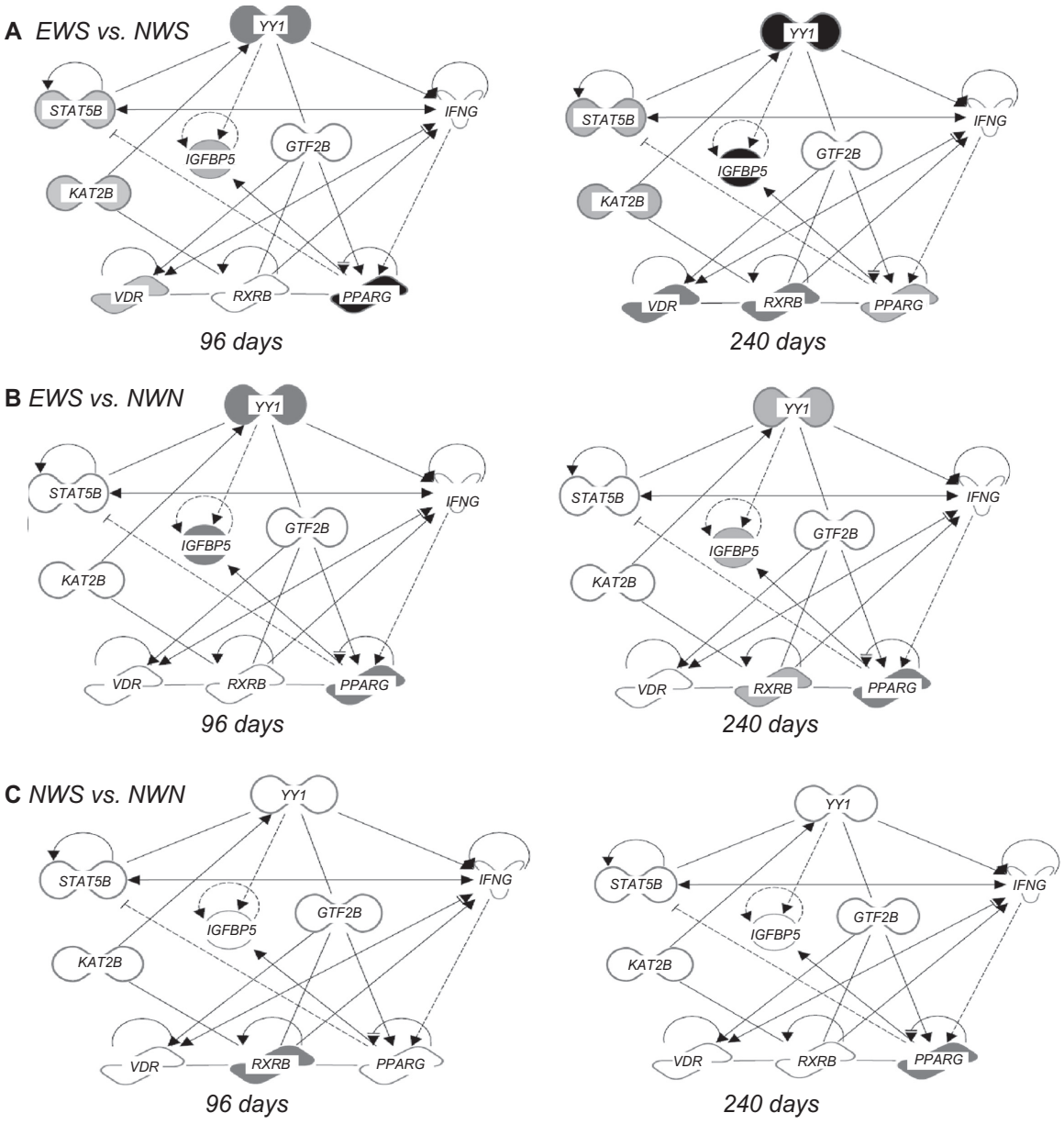

Figure 3. YY1 and target gene networks generated using Ingenuity Pathway Analysis®.
Notes: Shown at each time point are up-regulated (black to grey background according with the level of expression; black color (highly expression) to light gray color (slightly expressed). Genes with white-colored background were not affected significantly by the interaction of diet × time. Arrows denote direct (solid lines) or indirect (dotted lines) interactions among genes.

steers to reach the point of harvest, EWS steers had longer access to a diet that is more typical of the finishing phase, ie, the molecular mechanisms driving marbling deposition were likely to have been activated earlier than normal, as we have previously seen.[6] These responses to high-starch feeding were accompanied by greater concentrations of leptin in serum. This response could partly explain the greater marbling in EWS steers during the finishing phase as serum leptin levels are positively correlated with percentage of body fat and fat mass. Steers in these groups likely had larger adipocytes, which actively secrete leptin.[13]

Early studies led to the suggestion that calves must be creep-fed for ca. 80 days to express their maximum potential for marbling deposition.[8] However, despite NWS steers receiving creep feed for a period of 96 days, that in itself did not seem to affect the molecular mechanisms enhancing marbling relative to steers which received milk and pasture as their sole source of feed (NWN). Our results underscored the potential for precocious initiation of marbling in young steers when fed higher dietary starch.

Yin Yang 1 network gene expression

Although *PPARG* is not a direct target of YY1 (Fig. 3), YY1 binds and represses *PPARD* leading to inhibition of ligand-induced transcription activity.[14] Thus it is possible that YY1 in bovine skeletal muscle exerts some degree of inhibition over *PPARG,* which seems to be supported by the contrasting response in expression of *PPARG* and YY1 specifically in the EWS group (Figs. 2 and 3). Those animals had greater *PPARG* expression, supporting the existence of a more robust adipogenic/lipogenic activity.

The bioinformatics analysis using IPA of microarray data from LM in our previous study[1] revealed that the complex formed between *VDR* and *RXR* is affected by YY1. Transcription Factor 2 B (*TFIIB*) and CREB binding protein (CBP) bind to YY1 and regulate activation of transcription via 1,25-$(OH)_2D_3$ through an alleviation of the repressive effect induced upon YY1 binding to *VDR*.[15] YY1 also competes for the vitamin D response element (VDRE) with *TFIIB* through protein-protein interactions.[16] Hence it has been speculated that maximizing suppression of vitamin D induction when a target gene is not transcriptionally active may enhance affinity of YY1 for VDRE.[17] The outcome of this suppression would be the maintenance of basal transcription and inhibition of vitamin D sensitivity.

In NIH3T3 adipocytes, there is a stimulation of YY1 expression, which is dependent on serum containing IGF-1. Moreover, after prolonged serum deprivation, NIH3T3 cells lost YY1 expression. The rapid change in YY1 expression due to growth factor deprivation suggested the possibility that YY1 mediates some of the intracellular responses to IGF-1.[18] This fact is important in the context of our study as it was previously shown that serum IGF-1 concentration in Angus steers had a negative correlation with greater marbling scores, quality grades, fat thickness, and yield grades.[19] Most IGF-1 in the circulation is bound to IGFBP, which can either inhibit or facilitate IGF-1 binding to the IGF-1 receptor. *IGFBP5* is a YY1 target gene in mouse embryonic fibroblasts (MEF) and it is expressed in white adipose tissue, kidney, and heart.[20] The pro-adipogenic role of *IGFBP5* in LM tissue is likely indirect (eg, Fig. 3) and via several VDRE and *PPARG* response elements (PPRE) previously discovered in *IGFBP5* promoter regions.[21]

IGF-1 is produced primarily by liver as an endocrine hormone stimulated by GH, and its effect can be delayed by undernutrition, GH insensitivity, lack of GH receptors, or failure of the GH receptor downstream signaling pathway that employs *STAT5B*.[22] Work in non-ruminants showed that YY1 is a component of the GH-inducible nuclear factor complex (GHINF), which exerts close regulation of *STAT5B* transcription.[23] Since the distribution and function of each STAT (*STAT* 1, 2, 3, 4, 5*A*, 5*B* and 6) is unique, the regulation of tissue specific genes may reflect a physiological role for these proteins.[24] In adipose tissue, *STAT5B* activation is highly-specific and, upon activation, only GH was able to induce *STAT5B* to translocate to the nucleus.[25] Interestingly, YY1 associates with *STAT5B* during the response to GH in adipose tissue.[23] GH-activated *STAT5B* can inhibit PPAR-regulated transcription. This inhibitory crosstalk is mutual, ie, GH-induced-*STAT5B* can be inhibited by ligand-activated *PPARG*.[26]

In the current study, the antagonistic transcriptional role of GH on *PPARG* has to be discarded as a central regulatory mechanism associated with marbling mainly because of a lack of treatment differences on *STAT5B* expression. Although it has been

hypothesized that STATs may have a regulatory function in adipocyte gene expression, their particular functions during adipogenesis or in the mature adipocyte remain mostly unknown. Results showed a modest but consistent downregulation of *STAT5B* expression during development, which may be partly explained by the upregulation (ie, stimulation) of *PPARG*. Therefore, the lack of treatment effect on *STAT5B* expression argues against a possible GH-mediated inhibition of *PPARG* activation by *STAT5B* which was reported previously in rodents.[26] Such a mechanism was most evident in the EWS group.

GTF2B/*TFIIB* (General transcription factor IIB) is one of the ubiquitous transcription factors required for transcription initiation [ie, formation of the pre-initiation complex (PIC)] by RNA polymerase II (RNAP II). The rate of RNAP II-directed transcription is affected by *VDR*.[27] YY1 represses *VDR*-mediated 25-Hydroxy-vitaminD_3-24-hydroxylase transcription by sequestering *TFIIB*/CBP. In addition, the N-terminal region of cAMP response element-binding protein (CBP) that interacts with YY1 can inhibit YY1 from binding to *TFIIB*. Thus, CBP may diminish YY1-mediated repression by preventing YY1 from binding to *TFIIB*, which is required for *VDR*-mediated transcription.[15] Besides its role as lipogenic substrate, acetate availability in intramuscular adipocytes within LM of growing steers also could alter the function of *GTF2B*, which is an acetyltransferase with a key role in promoting gene transcription.[28]

One likely explanation for the greater *GTF2B* expression due to NWN is the higher availability of acetate (and acetyl-CoA) as substrate for the acetyltransferase reaction. That is, the pasture intake enhanced the production of ruminal acetate and could have enhanced transacetylation, thus, facilitating the interaction of *GTF2B* with other members of the PIC complex and SWI/SNF complex.[29] In that context, our data underscored that lipogenic substrate availability per se (ie, greater acetate) is insufficient to promote adipogenesis. In NWN steers, it is also possible that although the PIC complex was fully formed it remained "inactive" perhaps due to lower glucose availability (from gluconeogenesis) for ATP generation compared with EWS and NWS. The end result of such adaptations would have been a "repression" of the transcription machinery.

The transcriptional repressor activity of YY1 is regulated through histone acetylation by the transcription coactivators p300 (*KAT3B*) and PCAF (*KAT2B*).[30] PCAF-mediated acetylation supports SWI/SNF recruitment to the *PPARG* promoter[31] and functions as a nuclear receptor coactivator for retinoic acid receptor (*RAR*). Both *KAT2B* and *GTF2B*, encoding histone transacetylase, had a similar expression pattern in NWN steers likely due to greater availability of acetate and acetyl-CoA. However, as indicated above this response was insufficient to promote a pro-adipogenic response.

Summary Model

Figure 4 depicts a model based on data from the present study and data from published literature cited in the manuscript. The YY1-related network is represented by the genes encoding *IGFBP5*, *STAT5B, GTF2B*, and *KAT2B*. The transcription factor YY1 is part of the GHINF complex in which the major DNA binding component is *STAT5B*. Signaling via GH induces tyrosine phosphorylation, nuclear localization, and DNA binding of *STAT5B*. The GH-induced *STAT5B* signaling in primary preadipocytes represses transcription of genes required for terminal differentiation, partly by repressing *PPARG*. The *GTF2B*/*TFIIB* is one of the ubiquitous factors required for transcription initiation by RNA polymerase II. The interaction of the *VDR* with *TFIIB* represents a potential physical connection between the *VDR*-DNA complex and the transcription PIC. YY1 represses *VDR*-mediated 25-Hydroxy-vitaminD3-24-hydroxylase transcription by sequestering *TFIIB*/*CBP*.

The transcriptional PIC interacts with transcriptional co-activators via protein–protein interactions. These co-activators are an ATP-dependent chromatin remodeling complex called SWI/SNF that leads to local chromatin de-condensation and gene expression. The SWI/SNF complex has a role as a bromodomain-containing transcriptional co-activator/co-repressor in adipogenesis. The *KAT2B* protein has a role in ensuring the maintenance of a stable association of the complex with chromatin, hence, maintaining the PIC on the *PPARG* promoter to support transcription. YY1 corepressor activity is regulated through histone acetylation by PCAF (*KAT2B*). Acetylation of the central region of YY1 is required for a full transcriptional repressor activity, while acetylation of the

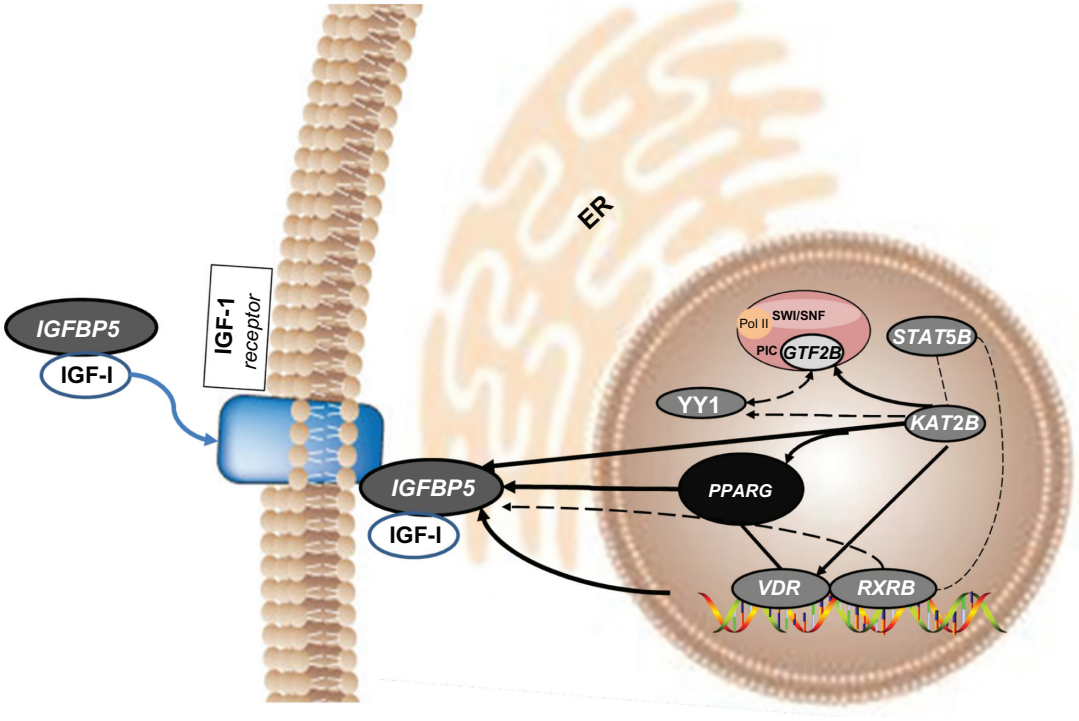

Figure 4. Summary of YY1 gene network expression in steers comparing early weaning (EWS) against normal weaning without creep feeding (NWN) at 240 days on treatment.
Notes: Cellular locations depicted are from the Ingenuity Pathway Analysis® knowledge base. Differences in color denote expression level of genes comparing EWS and NWN treatments (black to grey background according with the level of expression; black color (highly upregulated) to grey color (modest upregulation). Also shown are all significant ($P < 0.05$) positive correlations (thick continuous lines) and non-significant correlations (thin dotted lines) due to treatment × time in EWS and NWN.

C-terminal zinc finger domain decreased the DNA-binding activity of YY1.

Conclusions

Based on upregulation of *PPARG,* early exposure to a diet with a high-starch level appeared to induce precocious intramuscular adipocyte differentiation that led to greater marbling score in the carcass. As the response did not seem to involve YY1, its role in this process is likely minor. During the growing phase, diets that increase ruminal acetate to propionate ratio (NWN) seemed to induce formation of a transcriptional complex, suggesting that transacetylation of *GTF2B* is responsive to substrate availability. The availability of adequate propionate for gluconeogenesis and subsequent provision of glucose to LM also seems important in order to activate the transcriptional machinery.

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Author Contributions

Conceived and designed the experiments: JJL, DWS, DBF. Conceived and performed the analyses: SJM, WTM, DK. Wrote the manuscript: SJM, JJL. Agree with manuscript results and conclusions: SJM, DWS, WTM, DK, DBF, JJL. Jointly developed the structure and arguments for the paper: SJM, JJL. Made critical revisions and approved final version: SJM, DWS, JJL. All authors reviewed and approved of the final manuscript.

Competing Interests

Author(s) disclose no potential conflict of interest.

Disclosures and Ethics

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

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