BMB
reports

Regulation of ANKRD9 expression by lipid metabolic perturbations

Xiaofei Wang[1,2,], Robert F. Newkirk[1], Wilfrid Carre[2,3], Purnima Ghose[1], Barry Igobudia[1], James G. Townsel[4] & Larry A. Cogburn[2]*

[1]Department of Biological Sciences, Tennessee State University, 3500 John A Merritt Blvd, Nashville, TN 37221, [2]Department of Animal and Food Sciences, University of Delaware, Newark, DE 19716, USA, [3]Department of Genetics and Genomics, Roslin Institute, Roslin EH25 9PS, Scotland, UK, [4]Department of Physiology, Meharry Medical College, Nashville, TN 37208, USA

Fatty acid oxidation (FAO) defects cause abnormal lipid accumulation in various tissues, which provides an opportunity to uncover novel genes that are involved in lipid metabolism. During a gene expression study in the riboflavin deficient induced FAO disorder in the chicken, we discovered the dramatic increase in mRNA levels of an uncharacterized gene, ANKRD9. No functions have been ascribed to ANKRD9 and its orthologs, although their sequences are well conserved among vertebrates. To provide insight into the function of ANKRD9, the expression of *ANKRD9* mRNA in lipidperturbed paradigms was examined. The hepatic mRNA level of ANKRD9 was repressed by thyroid hormone (T_3) and fasting, elevated by re-feeding upon fasting. However, ANKRD9 mRNA level is reduced in response to apoptosis. Transient transfection assay with green fluorescent protein tagged- ANKRD9 showed that this protein is localized within the cytoplasm. These findings point to the possibility that ANKRD9 is involved in intracellular lipid accumulation. [BMB reports 2009; 42(9): 568-573]

INTRODUCTION

In the chicken, embryonic development in the second half is almost solely fueled by fatty acid oxidation (FAO). A strain of *rd/rd* chicken which lack riboflavin binding protein lays eggs deficient in riboflavin that is required for acyl-CoA dehydrogenases (1, 2). Lacking the required cofactor for these enzymes, FAO in the Rf-deficient embryos is severely impaired (3), leading to the abnormal hepatic accumulation of lipids. In these embryos, hepatic expression of *PPARγ* and adipophilin (ADFP) mRNA is elevated, accompanied by down regulation of the expression of protease inhibitor genes (reported separately). During an initial gene expression profiling in these embryos, we discovered that *ANKRD9* mRNA was dramatically elevated in the Rf-deficient (Rf^-) embryos around 13th day of incubation. The function of *ANKRD9* and its orthologs is not yet known. We reasoned that the elevation of *ANKRD9* mRNA is a response in order to accommodate surplus of fatty acids. Here, we report an examination of this possibility.

*Corresponding author. Tel: 1-615-9632541; Fax: 1-615-9625780; E-mail: xwang@tnstate.edu

Received 25 February 2009, Accepted 18 May 2009

Keywords: ANKRD9, Ankyrin repeat, Chicken, Fatty acid oxidation, Liver, Riboflavin deficient

RESULTS

ANKRD9 orthologs have an ankyrin repeat domain similar to calcium-independent phospholipase A_2 and are highly conserved among vertebrates

Microarray profiling of hepatic gene expression in riboflavin deficient chicken embryos revealed over-expression of ANKRD9 mRNA. A database search revealed that no function has been assigned to any orthologs of vertebrate ANKRD9. An invertebrate equivalent of ANKRD9 with significant sequence similarity (BLAST bit score $>$ 45) could not be identified from GenBank database. The chicken *ANKRD9* (*cANKRD9*) encodes a predicted peptide of 292 amino acids with a molecular mass of 32 kDa, while the ortholog in humans encodes a peptide of 317 amino acids (GenBank accession # AAH27479). Alignment of ANKRD9 orthologs indicates that this peptide is highly conserved in vertebrates (Fig. 1). It lacks an apparent signal peptide and transmembrane domain. The subcellular location of ANKRD9 cannot be unequivocally deduced by various prediction methods because it lacks a recognizable sorting signal that is recognized by WoLF PSORT (http://wolfpsort.seq.cbrc.jp/) or LOCtree (http://cubic.bioc.columbia.edu/cgi-bin/var/nair/loctree/query). ANKRD9 has an ankyrin repeat domain in the N-terminus. The most closely related protein of ANKRD9 is calcium-independent phospholipase A2 which has 44% identical amino acids to the central ankyrin repeat domain, but without a catalytic motif. No significant homology to other proteins could be identified in the N-terminus and C-terminus of ANKRD9.

Orthologs of *ANKRD9* are located at *Hsa14q32*.31, *Mmu12F1* and *Cga5* in human, mouse and chicken respectively. The human *ANKRD9 (hANKRD9)* has 4 exons with the entire peptide encoded in exon 4. Alignment of EST sequences in humans shows that *hANKRD9* mRNA has 7 alternative splicing variants.

We have examined Gene Expression Omnibus data at GenBank and Cancer Genome Anatomy SAGE. These expression data show that levels of hANKRD9 mRNA expression vary widely.

The chicken *ANKRD9 (cANKRD9)* also encodes the entire peptide by the last exon. The chicken mRNA is expressed in a variety of tissues, including heart, muscle, brain, and liver. We noticed that a locus on chicken chromosome 3 was also annotated as *cANKRD9* in Entrez Gene database (GenBank accession: XM_419365). However, its hypothetical peptide shares only 68% similar amino acid residues with the one on chromosome *Cga5* (represented by GenBank accession # XM_421377).

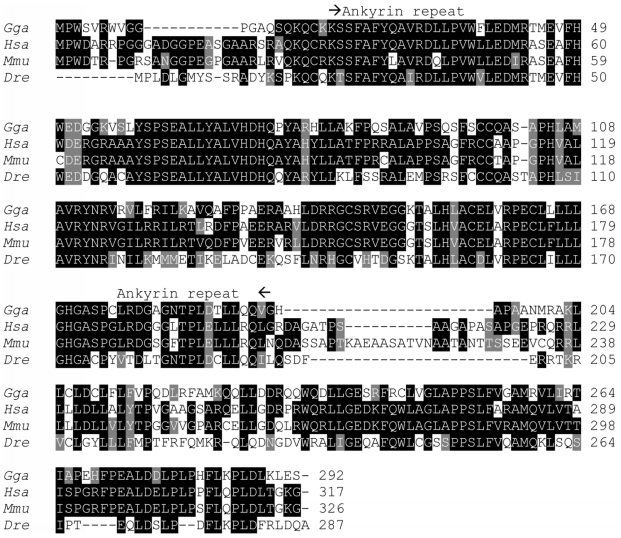

Fig. 1. Comparison of ANKRD9 amino acid sequences from chicken (*Cga*), human (*Hsa*), mouse (*Mmu*) and zebra fish (*Dre*). Amino acids are numbered on the right and identical or similar ones among species are highlighted in black or grey background, respectively, while dashes represent gaps. Overall similarities of human ANKRD9 to its orthologs in mouse, chicken and zebra fish are 85%, 70%, and 63%, respectively. The region between the two arrows marked on top of the sequences is the ankyrin repeat domain.

ANKRD9 mRNA levels are responsive to lipid metabolic perturbation *in vivo*

Data obtained by our earlier gene expression profiling indicate the over-expression of *cANKRD9* in Rf-deficient chicken embryo. A qRT-PCR analysis confirmed our microarray observation. We examined hepatic mRNA transcript abundance in Rf-rescued (control) and Rf-deficient embryos at embryonic age (e) 9, 11, 13 and 15 days of incubation with qRT-PCR analysis. Chicken hepatic *cANKRD9* transcripts were dramatically increased by 12- and 6.4-fold at e13 and e15 (respectively) in Rf-deficient (Rf^-) embryos compared with age-matched Rf-rescued (Rf^+) embryos (Fig. 2A). We reasoned that the increase in ANKRD9 could result from intracellular lipid accumulation. Therefore, we examined whether lipid metabolic perturbations are associated with changes in cANKRD9 mRNA abundance. Thyroid hormone T_3 increases energy expenditure (4, 5), reduces hepatic triglyceride content (6), and decreases body fat content (7). Chickens treated with dietary T_3 showed decreased mRNA level of cANKRD9, compared with chickens fed the control diet (Fig. 2B).

Next, we examined cANKRD9 mRNA levels in chickens during fasting and refeed. Random grouped chickens were subjected to 5 feeding treatments: (1) fully fed, (2) fasted for 16 h, (3) fasted for 48 h, (4) refeed for 4 h following 48-h-fast, and (5) re-feed for 24 h following 48-h-fast. Hepatic cANKRD9

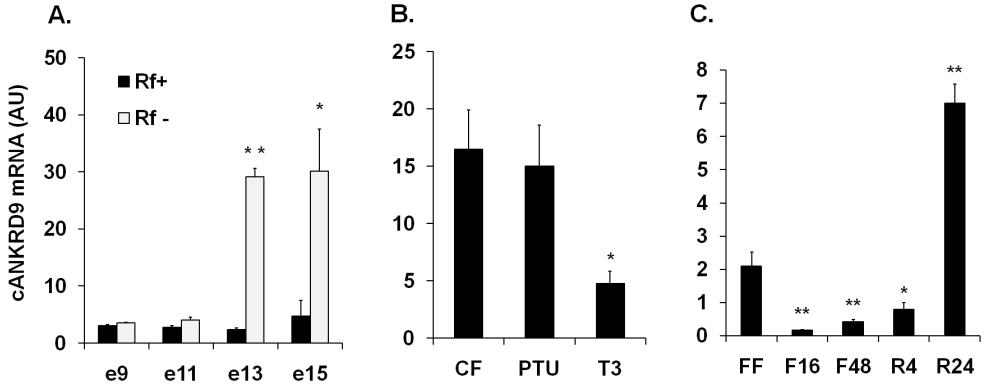

Fig. 2. Regulation of ANKRD9 mRNA levels in chickens. (A) ANKRD9 mRNA levels in the liver of chicken embryos. Rf-deficient eggs were injected with FMN (Rf^+) or saline (Rf^-) before incubation and embryos were collected at embryonic age (e) indicated. Asterisks indicate significant differences in mRNA levels between riboflavin deficient embryos (Rf^-) and age-matched riboflavin rescued embryos (Rf^+) (*$P < 0.05$; **$P < 0.01$). (B) Hepatic ANKRD9 mRNA levels in thyroid hormone manipulated chickens. Juvenile cockerels were given control feed (CF), dietary 0.5% propylthiouracil (PTU) or dietary triodothyronine (T_3) for two weeks before collection of livers for qRT-PCR analysis. Asterisk denotes significant difference from the control feed group (*$P < 0.01$). (C) Hepatic mRNA levels in a slow growing strain of chickens after fasting and re-feeding [FF, fully fed; F16, fast for 16 h; F48, fast for 48 h; R4, fed 4 h following 48 h fast; and R24, fed 24 h following 48 h fast]. Asterisks denote significant differences from the fully fed group (*$P < 0.05$; **$P < 0.01$). There were four animals in each treatment group.

mRNA levels were reduced in fasting birds when compared to fully fed birds. The reduction in mRNA level was not fully recovered at 4 h after re-feeding, but was over expressed at 24 h after re-feeding (Fig. 2C). This mRNA expression pattern of ANKRD9 is similar to that of PPARδ and PPARγ, but in contrast to that of PPARα [see Fig. 4 in ref (8)].

Chicken ANKRD9 mRNA level is reduced by geneticin-induced apoptosis but less responsive to free fatty acid

On day 13 of incubation, Rf-deficient embryos exhibited many secondary responses, including apoptotic cell death, to the lipid metabolic crisis. To investigate the possibility that the overexpression of ANKRD9 mRNA is mediated through the apoptotic pathway, we analyzed mRNA levels in two cell lines following apoptosis induction. HepG2 cells were treated with or without G418, a cell death inducer through the apoptosis pathway (9). As shown in Fig. 3A, upon 48 h treatment, the mRNA level of caspase 3 (CASP3), a molecular marker of apoptosis (10), was increased in a dose-dependent manner, suggesting apoptosis was induced following G418 treatment. However, the mRNA levels of *hANKRD9* and adipophilin (ADFP) were markedly reduced. Similar results were obtained in HEK 293 cells (data not shown).

Since alteration of *ANKRD9* mRNA levels is accompanied by lipid metabolic perturbations *in vivo,* and PPARγ is a potent regulator of lipid accumulation, it was of interest to clarify whether *ANKRD9* transcription is enhanced by the activation of PPARγ. We investigated *hANKRD9* mRNA levels in HepG2 and HEK 293 cells treated with or without oleic acid, an activator of PPARγ (11). As shown in Fig. 3B, mRNA level of adipophilin (ADFP), a target gene of PPARγ and indicator of intracellular lipid load (12, 13), was up-regulated in a dose dependent manner, while the level of caspase 3 (CASP3) mRNA was not changed by oleic acid treatment. The levels of hANKRD9 mRNA showed a tendency of increasing.

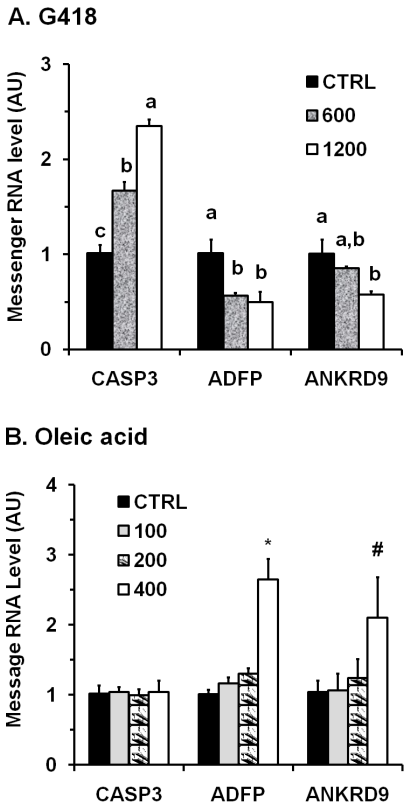

Fig. 3. Response of caspase 3 (CASP3), adipophilin (ADFP) and ANKRD9 mRNA levels to geneticin-induced apoptosis and oleic acid-induced lipid accumulation. (A) HepG2 cells were treated with geneticin G418 (0 [CTRL], 600, and 1,200 μg/ml) for 48 h. Total RNA was analyzed with qRT-PCR (n = 4, $^{a,b,c}P < 0.05$ between any two bars of the same gene with no common scripts by ANOVA). Data are representative of two independent experiments. (B) HepG2 cells were treated with oleic acid (0 [CTRL], 100, 200, and 400 μg/ml) for 24 h (n = 4; *P < 0.05 between control and $^{\#}P = 0.13$ between control by *t*-test).

GFP-tagged ANKRD9 is not diffused in cells cultured *in vitro*

Subcellular location of a protein provides important information about its possible function. We attempted to locate the subcellular structure where ANKRD9 may assume its role. Two expression vectors were constructed in which green-fluorescent

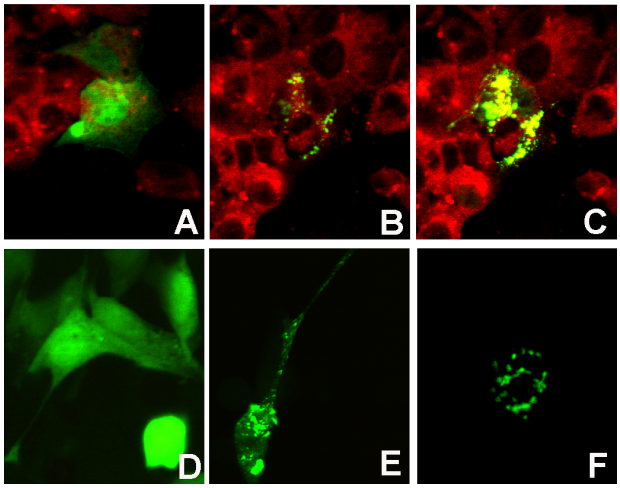

Fig. 4. Subcellular localization of ANKRD9-GFP fusion protein. Cells were transiently transfected with GFP expression vector alone (A, D), human hANKRD9- GFP fusion construct (B, C) or chicken cANKRD9-GFP fusion construct (E, F) for 24 h. HepG2 cells were counterstained with BODIPY ceramide (A-C). HEK 293 cells (D, E) and COS7 cells (F) were not counterstained. The fusion protein appears to be restricted to particle-like structures around the nucleus or other regions in the cytoplasm of most transfected cells. The green fluorescence was much brighter in most fusion-construct-transfected cells than that in GFP-vector-alone-transfected cells. In order to show the detail of green fluorescence in fusion construct transfected cells, two images were captured under full argon laser power (C) and attenuated laser power (B). Full laser power was used to capture images for GFP-alone- transfected cells.

protein (GFP) was fused with the N-terminus of cANKRD9 or hANKRD9 coding sequence. The hANKRD9-GFP constructs were used to transfect human cell lines HepG2 and HEK 293. The cANKRD9-GFP constructs were used to transfect a COS7 cell line and a quail cell line QNR/D. The subcelluar locations of the GFP and fusion proteins were examined with confocal microscopy. As shown in Fig. 4, GFP alone was diffuse in the cytoplasm and nucleus in cells transfected with the control plasmid (Fig. 4A, D), while hANKRD9-GFP and cANKRD9-GFP fusion proteins are restricted to the cytoplasm and form particle-like structures (Fig. 4B, C, E, F) in most transfected cells. These particles do not colocalize with Golgi complexes, which were bright red after BODIPY ceramide stain (Fig. 4A-C). Similar results were obtained when HepG2 (not shown), HEK 293 (Fig. 4D, E), COS7 (Fig. 4F) or QNR/D (data not shown) cells were transfected with cANKRD9- GFP constructs.

DISCUSSION

The riboflavin-deficient chicken embryo is a model for the study of fatty acid oxidation disorders. In these embryo, Rf-deficiency results from a defect in riboflavin binding protein in hens (2). Riboflavin, in the form of flavin adenine dinucleotide (FAD) or FMN, is a cofactor of chain-length-specific acyl-CoA dehydrogenases and electron transferring flavoprotein. In this chicken embryo model, Rf-deficiency primarily impairs mitochondrial FAO with symptoms similar to inherent defects of FAO enzymes, for example, very long chain and medium chain fatty acyl dehydrogenases (3). Rf-deficient embryos die between 13-15 days of incubation if they are not rescued with riboflavin (14). A devastating pathological consequence of Rf-deficiency in the chicken embryo is the depletion of carbohydrate reserves (14, 15). The inability of Rf-deficient embryos to switch to fatty acid oxidation is reflected by decreased activity of MCAD and abnormal accumulation of intermediate (C_8, C_{10} and C_{12}) substrates (3). Histological examination of the Rf-deficient liver prior to death revealed an accumulation of lipid droplets in hepatocytes. This is accompanied by the up-regulation of genes that promote intracellular lipid storage, including adipophilin, PPARγ. Thus, it is possible that an increase in ANKRD9 in Rf-deficient embryos around 13 days of incubation is a response to lipid perturbation. We then examined ANKRD9 mRNA expression in chickens fasted or manipulated for thyroid status. Increased plasma thyroid hormone reduces abdominal fat, as well as ADFP expression (7). Similarly, reduction in the expression of ANKRD9 was also seen in thyroid manipulated chickens. During fasting, hepatic expression of fatty acid utilization genes, for example PPARα, was increased and expression of genes that promote lipid storage was decreased (8). Accordingly, ANKRD9 mRNA was reduced. These observations are consistent with the assumption that ANKRD9 participates in lipid metabolism. Interestingly, ANKRD9 is located within a quantitative trait locus for abdominal fat on *Cga5* in the chicken (16). It is less likely that the increased expression of ANKRD9 mRNA is a response to apoptotic cell death because induced apoptosis *in vitro* did not lead to an elevation of ANKRD9 mRNA.

Another line of evidence that hints ANKRD9 in lipid metabolism comes from the sequence analysis. ANKRD9 has an N-terminal ankyrin repeat domain which in many cases is involved in protein-protein interactions (17). Ankyrin-repeat-domain-containing proteins are involved in various cellular functions, including intracellular adaptor (18, 19), trafficking (20, 21), transcription (22, 23), signal transduction (24) and stress response (25). The ANKRD9 ankyrin repeat domain showed the highest sequence similarity to calcium-independent phospholipase A2 (iPLA2), an enzyme that catalyzes the release of fatty acid from phospholipids [reviewed in (26, 27)]. Because ANKRD9 does not have the lipase activity signature site GXSXG, it is unlikely to have lipase activity. Interestingly, the human iPLA2 has several splicing isoforms. Two isoforms also lack a catalytic site (28). The function of these isoforms is unknown. This sequence feature may render ANKRD9 as a molecular chaperone. Nevertheless, alterations of ANKRD9 expression by lipid perturbations strongly suggest that ANKRD9 does play a role in vertebrate lipid metabolism.

MATERIALS AND METHODS

Animals and RNA samples

A strain of Rf-binding protein-deficient (rd/rd) single comb white Leghorn chickens (2) is maintained at the University of Delaware. Fertilized eggs were collected from the *rd/rd* hens and held at 8-12°C until setting. Prior to incubation, Rf-deficient eggs were randomly assigned to two treatment groups that were either injected with 0.2 mL flavin mononucleotide (FMN, 5.4 mg/mL) dissolved in 0.9% sterile saline (Rf^+, rescued control) or saline (Rf^-) alone into the egg white. After sealing the injection site with glue (Duco® Cement), the eggs were incubated at 39°C and 95% relative humidity. Liver samples were collected from 4 to 10 live embryos on embryonic (e) day e9, e11, e13 and e15. Tissues were rapidly frozen in liquid nitrogen and stored at −80°C until isolation of total RNA. The use of animals in these studies complies with the University of Delaware Animal Care and Use Guidelines. Experimental paradigms for thyroid hormone manipulation and nutritional management were described previously (7, 8, 29). Total RNA samples were isolated using RNeasy mini kit (Qiagen, Valencia, CA).

Real time RT-PCR

Messenger RNA levels were measured by real time quantitative RT-PCR (qRT-PCR) using Quantitect SYBR green RT-PCR kit (Qiagen). Total RNA was diluted in RNase-free water to a concentration of 50 ng/μl. The concentration was reassessed with Nanodrop spectrophotometer after dilution. Each reaction mix (20 μl) contained 100 ng of total RNA, 0.5 μM primers and 10 μl of master mix. For each sample, duplicated re-

actions were conducted. All real time RT-PCR primers were designed using Primer Express 2.0 (Applied Biosystems, CA). Sequences of the primers used are available upon request. Thermal cycles were setup following the manufacturer's recommendation. For every experiment, a melting curve analysis was performed. The level of mRNA is represented as $2^{-\Delta Ct}$, where the ΔCt is the difference between threshold cycle (Ct) of the sample and the Ct value that was used as one arbitrary unit of RNA.

Cell culture and treatment

Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/ml of streptomycin and 100 U/ml penicillin. Confluent cells were detached with 0.25% trypsin. For treatment with oleic acid, cells were seeded in 12-well plates. Stock oleic acid was prepared by dissolving in ethanol and then mixing with 10% bovine serum albumin to 10 μg/ml. Cells at confluent (2-3 days after plating) were treated with oleic acid at concentrations indicated. Geneticin concentration and times of treatment are indicated in Fig. 3B.

ANKRD9 construct

The coding sequence of chicken ANKRD9 mRNA was amplified using forward (5′-CAGTCGCAGAAGCAGTGCA-3′) and reverse (5′-CGCAGTACAGACCTCACCTCAC-3′) primers. This amplified region of ANKRD9 encodes the entire chicken ANKRD9 except the first 13 amino acids at the N-terminus. The PCR product was cloned into a pcDNA3.1/NT-GFP-TOPO vector (Invitrogen) which contains a CMV promoter. The correct orientation was verified by sequencing. To clone the human ANKRD9 coding sequence, the primers were: forward 5′-CC ATGCCTTGAGGCCCA-3′ and reverse 5′-AGGGTGCTCCGGG CCTA-3′. This segment encodes the entire human ANKRD9 and 12 additional amino acid residues at the N-terminus. The segment was also cloned in a pcDNA3.1/NT-GFP-TOPO vector.

Transfection and confocal microscopy

Cells were cultured in DMEM supplemented with 10% FBS, penicillin, and streptomycin. At 24 h before transfection, cells were plated in 4-well culture slides (BD, Franklin Lakes, NJ) at 1×10^5 per well. Transfection was carried out using lipofectamine 2,000 (Invitrogen, Carlsbad, CA) with 2 μg of either ANKRD9-GFP construct or GFP plasmid alone. At 24 h after transfection, cells were stained with 5 μM BODIPY TR ceramide (Invitrogen) for 30 minute followed by a PBS wash and fixed with freshly prepared 1% paraformaldehyde in PBS for 15 min. The slide was mounted with ProLong antifade reagent (Molecular Probes, Eugene, Oregon, USA). Images were captured using a Nikon PCM 2,000 confocal microscope with an argon laser (for green fluorescence) and a He/Ne laser (for red fluorescence). Emission filters were 515/530 band pass and 565 LP. In order to prevent over excitation in fusion construct transfected cells, some images were captured under attenuated laser power as indicated in figures.

Acknowledgements

This research was supported in part by NIH grant P20MD-000261(RFN, XW) and USDA grant (to LAC). We thank Dr. Michel Duclos of INRA, France for his kind gift of RNA samples. We are grateful to Dr. Harold White III, Department of Chemistry and Biochemistry at University of Delaware, and Dr. Brenda McAdory for their critical discussion and review of the manuscript. We thanks Dr. Dafeng Hui for assistance with ANOVA analysis. We also thank Ms. Spojmay Telware for her assistance in data collection.

REFERENCES

1. White, H. B., 3rd, Nuwaysir, E. F., Komara, S. P., Anderson, D. A., Chang, S. J., Sherwood, T. A., Alphin, R. L. and Saylor, W. W. (1992) Effect of riboflavin-binding protein deficiency on riboflavin metabolism in the laying hen. *Arch. Biochem. Biophys.* **295,** 29-34.
2. MacLachlan, I., Nimpf, J., White, H. B., 3rd and Schneider, W. J. (1993) Riboflavinuria in the rd chicken. 5'-splice site mutation in the gene for riboflavin-binding protein. *J. Biol. Chem.* **268,** 23222-23226.
3. Abrams, V. A., Han, C. C. and White, H. B., 3rd (1995) Riboflavin-deficient chicken embryos: hypoglycemia without dicarboxylic aciduria. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **111,** 233-241.
4. Weiss, R. E., Murata, Y., Cua, K., Hayashi, Y., Seo, H. and Refetoff, S. (1998) Thyroid hormone action on liver, heart, and energy expenditure in thyroid hormone receptor beta-deficient mice. *Endocrinology.* **139,** 4945-4952.
5. Moreno, M., Lombardi, A., Beneduce, L., Silvestri, E., Pinna, G., Goglia, F. and Lanni, A. (2002) Are the effects of T3 on resting metabolic rate in euthyroid rats entirely caused by T3 itself? *Endocrinology.* **143,** 504-510.
6. Mukhopadhyay, D., Plateroti, M., Anant, S., Nassir, F., Samarut, J. and Davidson, N. O. (2003) Thyroid hormone regulates hepatic triglyceride mobilization and apolipoprotein B messenger ribonucleic Acid editing in a murine model of congenital hypothyroidism. *Endocrinology.* **144,** 711-719.
7. Wang, X., Carre, W., Saxton, A. M. and Cogburn, L. A. (2007) Manipulation of thyroid status and/or GH injection alters hepatic gene expression in the juvenile chicken. *Cytogenet. Genome Res.* **117,** 174-188.
8. Cogburn, L. A., Porter, T. E., Duclos, M. J., Simon, J., Burgess, S. C., Zhu, J. J., Cheng, H. H., Dodgson, J. B. and Burnside, J. (2007) Functional genomics of the chicken–a model organism. *Poult. Sci.* **86,** 2059-2094.
9. Jin, Q. H., Zhao, B. and Zhang, X. J. (2004) Cytochrome c release and endoplasmic reticulum stress are involved in caspase-dependent apoptosis induced by G418. *Cell. Mol. Life Sci.* **61,** 1816-1825.
10. Boatright, K. M. and Salvesen, G. S. (2003) Mechanisms of caspase activation. *Curr. Opin. Cell Biol.* **15,** 725-731.
11. Xu, H. E., Lambert, M. H., Montana, V. G., Parks, D. J., Blanchard, S. G., Brown, P. J., Sternbach, D. D., Lehmann, J. M., Wisely, G. B., Willson, T. M., Kliewer, S. A. and

Milburn, M. V. (1999) Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. *Mol. Cell.* **3,** 397-403.
12. Mishra, R., Emancipator, S. N., Miller, C., Kern, T. and Simonson, M. S. (2004) Adipose differentiation-related protein and regulators of lipid homeostasis identified by gene expression profiling in the murine db/db diabetic kidney. *Am. J. Physiol. Renal Physiol.* **286,** F913-921.
13. Gross, D. N., Miyoshi, H., Hosaka, T., Zhang, H. H., Pino, E. C., Souza, S., Obin, M., Greenberg, A. S. and Pilch, P. F. (2006) Dynamics of lipid droplet-associated proteins during hormonally stimulated lipolysis in engineered adipocytes: stabilization and lipid droplet binding of adipocyte differentiation-related protein/adipophilin. *Mol. Endocrinol.* **20,** 459-466.
14. Lee, C. M. and White, H. B., 3rd (1996) Riboflavin-binding protein induces early death of chicken embryos. *J. Nutr.* **126,** 523-528.
15. White, H. B., 3rd (1996) Sudden death of chicken embryos with hereditary riboflavin deficiency. *J. Nutr.* **126,** S1303-1307.
16. Abasht, B., Pitel, F., Lagarrigue, S., Le Bihan-Duval, E., Le Roy, P., Demeure, O., Vignoles, F., Simon, J., Cogburn, L., Aggrey, S., Vignal, A. and Douaire, M. (2006) Fatness QTL on chicken chromosome 5 and interaction with sex. *Genet. Sel. Evol.* **38,** 297-311.
17. Mosavi, L. K., Cammett, T. J., Desrosiers, D. C. and Peng, Z.-y. (2004) The ankyrin repeat as molecular architecture for protein recognition. *Protein Sci.* **13,** 1435-1448.
18. Davis, L. H., Otto, E. and Bennett, V. (1991) Specific 33-residue repeat(s) of erythrocyte ankyrin associate with the anion exchanger. *J. Biol. Chem.* **266,** 11163-11169.
19. Nicolas, V., Le Van Kim, C., Gane, P., Birkenmeier, C., Cartron, J. P., Colin, Y. and Mouro-Chanteloup, I. (2003) Rh-RhAG/ankyrin-R, a new interaction site between the membrane bilayer and the red cell skeleton, is impaired by Rh(null)-associated mutation. *J. Biol. Chem.* **278,** 25526-25533.
20. Rader, K., Orlando, R. A., Lou, X. and Farquhar, M. G. (2000) Characterization of ANKRA, a novel ankyrin repeat protein that interacts with the cytoplasmic domain of megalin. *J. Am. Soc. Nephrol.* **11,** 2167-2178.
21. Zhang, X., He, X., Fu, X. Y. and Chang, Z. (2006) Varp is a Rab21 guanine nucleotide exchange factor and regulates endosome dynamics. *J. Cell Sci.* **119,** 1053-1062.
22. Sachdev, S., Hoffmann, A. and Hannink, M. (1998) Nuclear localization of IkappaB alpha is mediated by the second ankyrin repeat: the IkappaB alpha ankyrin repeats define a novel class of cis-acting nuclear import sequences. *Mol. Cell. Biol.* **18,** 2524-2534.
23. Zhang, A., Li, C. W., Tsai, S. C. and Chen, J. D. (2007) Subcellular localization of ankyrin repeats cofactor-1 regulates its corepressor activity. *J. Cell Biochem.* **101,** 1301-1315.
24. Lishko, P. V., Procko, E., Jin, X., Phelps, C. B. and Gaudet, R. (2007) The ankyrin repeats of TRPV1 bind multiple ligands and modulate channel sensitivity. *Neuron.* **54,** 905-918.
25. Miller, M. K., Bang, M. L., Witt, C. C., Labeit, D., Trombitas, C., Watanabe, K., Granzier, H., McElhinny, A. S., Gregorio, C. C. and Labeit, S. (2003) The muscle ankyrin repeat proteins: CARP, ankrd2/Arpp and DARP as a family of titin filament-based stress response molecules. *J. Mol. Biol.* **333,** 951-964.
26. Winstead, M. V., Balsinde, J. and Dennis, E. A. (2000) Calcium-independent phospholipase A2: structure and function. *BBA Mol. Cell Biol. Lipids.* **1488,** 28-39.
27. Adibhatla, R. M. and Hatcher, J. F. (2008) Phospholipase A(2), reactive oxygen species, and lipid peroxidation in CNS pathologies. *BMB Rep.* **41,** 560-567.
28. Larsson, P. K., Claesson, H. E. and Kennedy, B. P. (1998) Multiple splice variants of the human calcium-independent phospholipase A2 and their effect on enzyme activity. *J. Biol. Chem.* **273,** 207-214.
29. Beccavin, C., Chevalier, B., Cogburn, L. A., Simon, J. and Duclos, M. J. (2001) Insulin-like growth factors and body growth in chickens divergently selected for high or low growth rate. *J. Endocrinol.* **168,** 297-306.