Effects of fasting and refeeding on histone acetylation and related gene transcriptsin Xenopus laevis intestine

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ABSTRACT

Lower vertebrates, including amphibians, tolerate food deprivation for relatively long periods by depressing metabolic rate and can recover quickly from such a dormant state. In a previous chromatin immunoprecipitation (ChIP) study, we revealed the involvement of histone post-translational modifications in transcriptionally active genes in the intestine of adult *Xenopus laevis* under these stress conditions. To understand the molecular mechanism underlying these responses, we investigated whole cellular and gene-specific changes in the amounts of acetylated histone H3 at lysine 9 (H3K9Ac), trimethylated histone H3 at lysine 9 (H3K9me3), trimethylated histone H3 at lysine 36 (H3K36me3) and acetylated histone H4 (H4Ac)in fed, fasted and refed frogs by Western blotting and ChIP assay, respectively. Although there was no significant difference in the whole cellular levels of histone acetylation among the groups, the amounts of acetylated histones on three active genes known to down-regulated with fasting increased with fasting and decreased with refeeding. Transcript levels of histone acetyltransferases (HATs) and deacetylases (HDACs) declined with fasting, whereas those of one HAT (kat2a) and three HDACs (hdac1, hdac3 and sirt1) recovered with refeeding. Our results indicate that the amounts of acetylated histones detected in the intestinal genes of the fasted frogs may be increased by either the transcriptional down-regulation of HDAC genes or by other regulating factors at non-transcriptional levels.

Keywords: Fasting, histone acetylation, intestine, *Xenopus laveis*.

Abbreviations: ChIP: Chromatin immunoprecipitation; **H3K9ac:** Acetylated histone H3 lysine 9; **H3K9me3:** trimethylated histone H3 lysine 9; **H3K36me3:** trimethylated histone H3 lysine 36; **H4ac:** acetylated histone H4; **HAT:** histone acetyltransferase; **HDAC:** Histone deacetylase; **TBS:** Tris-buffered saline; **RT-qPCR:** Reverse transcription-quantitative polymerase chain reaction; **SDS:** sodium dodecylsulfate; **SEM:** standard error of the mean; **SIRT:** sirtuin.

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INTRODUCTION

Vertebrate ectotherms including amphibians can survive without food for relatively longer periods than endotherms, such as birds and mammals. Ectotherms have at least one order of magnitude lower standard metabolic rate than endotherms, which is beneficial for saving energy even in a fed state. During fasting, another important survival strategy for ectotherms is metabolic rate depression, the biochemical adaptation in response to food deprivation (Storey, 2015), accompanied by high plasticity in intestinal structure and function (Pennisi,

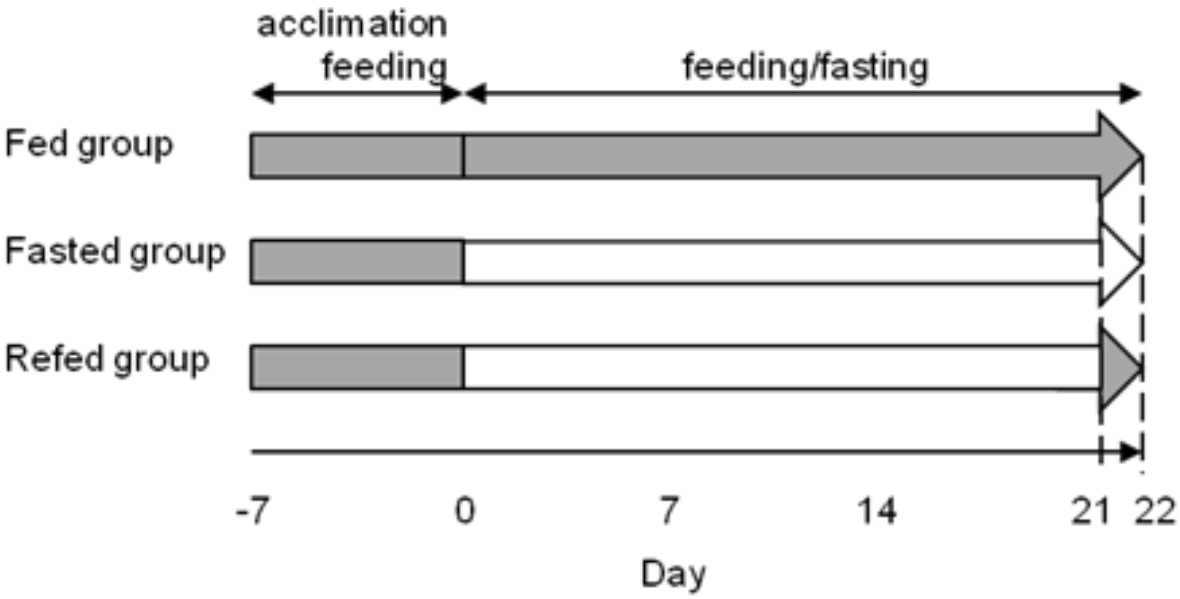

Figure 1: Experimental design. Adult male *Xenopus laevis* were fed 4 crickets/frog every day and were acclimated for 7 days at 23 ± 1°C before Day 0. Twenty-four frogs were divided into 3 groups (8 frogs/group): 1) normally fed crickets for 22 days (*fed group*), 2) fasted for 22 days (*fasted group*), 3) fasted for 21 days and refed Day 21 for 1 day (*refed group*). Gray shading indicates feeding; white shading indicates fasting.

2005; Zaldúa and Naya, 2014). Metabolic rate suppression is well known to occur in animals under environmental stress conditions, such as hypoxia [in squids (Seibel et al., 2014)], anoxia [in turtles (Krivoruchko and Storey, 2015) and hibernation [in ground squirrels (Morin and Storey, 2006; Biggar and Storey, 2014)], where the amounts of histone modifications are changed at a whole cellular level. However, the mechanism underlying this adaptive response remains to be elucidated at the cellular and molecular levels.

In a previous study, we investigated the morphological, biochemical and transcriptional changes in the intestine from fed, fasted and refed *Xenopus laevis* (Tamaoki et al., 2016). The weight of the intestine from frogs fasted for 3 weeks was approximately half of that from fed frogs. Although atrophy of the intestinal epithelial layer was remarkable, its structure remained to be intact. Transcriptome analysis of 74 genes revealed overall suppression with fasting, indicating metabolic rate depression, and quick recovery with subsequent 1-day refeeding. An unexpected finding was a reverse relationship between the transcript levels and the amounts of euchromatin-associated epigenetic marks found in histones (methylation and acetylation) and RNA polymerase II (phosphorylation) in the coding regions of five genes. This finding was in contrasted with those for rat small intestine (Ogura et al., 2007; Suzuki et al., 2011, 2008). Although it is likely that this reverse relationship reflects one of the adaptive responses to the extreme fluctuations in food availability, its molecular mechanism is unclear.

In this study, we assess how histone acetylation levels increase with fasting and recover with refeeding. We first examined the relative amounts of whole cellular histone modifications, acetylation levels of histones H3 at lysine 9 (H3K9ac) and H4 (H4ac) and trimethylation levels of H3 at lysine9 (H3K4me9) and at lysine 36 (H3K36me3), by Western blotting, and compared them with the relative amounts of histone modifications in specific genes investigated by chromatin immunoprecipitation (ChIP) assay. We further quantified the transcript amounts for histone acetyltransferases (HATs), and deacetylases (HDACs) and some other factors known to be important for nutritional deprivation by real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

MATERIALS AND METHODS

Animals and treatment

Adult male *Xenopus laevis* (age: 1 year, weight: 51 to 54 g)were obtained from Watanabe Breeding, Hyogo, Japan. Frogs were kept in 15 L of aerated and dechlorinated tap water at 23 ± 1°C and fed 4 middle-sized crickets/frog everyday. After 7 days of acclimation, 24 frogs were divided into 3 aquaria (8 frogs/aquarium) (Figure 1). Frogs in the first aquarium were fed crickets for 22 days as done previously (fed group) (Tamaoki et al., 2016). Frogs in the second aquarium were fasted for 22 days (fasted group) and frogs in the third aquarium were fasted for 21 days and refed on Day 21 (refed group). The water in the aquaria was changed three times a week. On Day 22, frogs were decapitated, and their intestines were collected for ChIP assay, Western blotting and RNA

preparation.

All animals were cared for under the international guidelines "Act on Welfare and Management of Animals" (Ministry of Environment of Japan) and all the experimental procedure shad approval of the Animal Welfare Committee of Shizuoka University.

ChIP assay

ChIP assays for histone modifications on frog intestine were performed as described previously (Tamaoki et al., 2016) with rabbit antibodies against H3K36me3 (ab9050, Abcam, Tokyo, Japan), H3K9me3, H3K9ac, H4ac, human histone H3 pan andhistone H4 pan (07-442, 17-615, 06-598, 07-690 and 05-858, respectively, Merck Millipore, Darmstadt, Germany). A normal rabbit IgG was also used as a negative control. In brief, after the lumen was washed with frog Ringer solution, the intestines were fixed with 1% formaldehyde solution for 15 min at 25°C. The crosslink reaction was terminated by the addition of glycine at the final concentration of 150 mM, followed by washing with FACS solution (1 × PBS, 2% bovine serum and 0.05% NaN_3). The samples were solubilized and then sonicated in SDS-lysis buffer [50 mM Tris-HCl, pH 8.0, 10 mM EDTA and 1% sodium dodecylsulfate (SDS)] to prepare appropriately sized DNA (200 to 500 bp). For immunoprecipitation, the protein concentration of the chromatin was diluted to 1 mg/mL with ChIP dilution buffer (50 mM Tris-HCl, pH 8.0, 167 mM NaCl, 1.1% Triton X-100and0.11% sodium deoxycholate). After the chromatin sample (1 ml) was pre-cleaned with salmon sperm DNA/Protein G-Sepharose beads (10 μl; 50% Protein G-Sepharose, 100 μg/ml salmon sperm DNA and 1% bovine serum albumin), input samples (50 μl) were withdrawn, and each chromatin sample (225 μl) was immunoprecipitated with one of the indicated antibodies (0.3 to 0.6 μg) or normal IgG (3.0 μg) and salmon sperm DNA/Protein G-Sepharose beads (5 μL).The immunoreactive chromatin was washed with RIPA buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.1% SDS and 0.1% sodium deoxycholate)/150 mM NaCl, RIPA buffer/500 mM NaCl, and LiCl buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% Nonidet P40 and 0.5% sodium deoxycholate), and then recovered in 400 μL of ChIP direct elution buffer (10 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5 mM EDTA and 0.5% SDS). Cross-linking reversal was done at 65°C overnight. After extraction with phenol/chloroform, the precipitated DNA was analyzed by real-time PCR on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using primers corresponding to the indicated sites in the coding region of the fabp1, cdx2 and fxr genes (Table 1). The Ct values for the ChIP signals detected by real-time PCR were converted to the percentage of each ChIP signal for input DNA by the $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001). Each ChIP experiment was done at least twice with similar results.

Western blotting

Intestine was homogenized in 4 volumes of 10 mM potassium phosphate buffer, pH 7.0, using a Potter-Elvehjem homogenizer, as previously shown (Tamaoki et al., 2016). Proteins in homogenates (60 μg) were electrophoresed in a 15% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to apolyvinylidene difluoridemembrane (0.22 μm, FluoroTrans®; PALL, Port Washington, New York, USA). The membrane was immersed in Tris-buffered saline (TBS, 50 mM Tris-HCl, pH, 7.6, and 140 mM NaCl) containing 10% of nonfat milk overnight. After blocking, the membrane was immunoblotted for 1 h at room temperature with rabbit antibodies against either H3K36me3, H3K9me3, H3K9ac, H4ac, human histone H3 pan or histone H4 pan (1:1000, 1:500,1:20000, 1:500, 1:25000 and 1:30000, respectively)in TBS containing 1% nonfat milk.The membrane was rinsed with TBS containing 0.1% Tween-20, and then incubated with the secondary antibody (1:2500, alkaline phosphatase-linked anti-rabbit immunoglobulin, from goat) in TBS containing 1% nonfat milk. Proteins transferred to the polyvinylidene difluoridemembrane were visualized by using a detection kit for alkaline phosphatase activity (ProtoBlot, Promega). Band intensity was quantified using an image analyzer (LAS-4000, GE Healthcare Life Sciences, Chicago, IL, USA).

Total RNA extraction and real-time RT-qPCR analysis

Total RNA was extracted from the intestines by the acid guanidinium-thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). RNA was resuspended in diethylpyrocarbonate-treated water and quantified with a UV spectrophotometer.RNA (0.2 μg) was treated with reverse transcriptase (Taqman RT Reagents Kit, Applied Biosystems) in 10 μl of 1 × Taqman RT buffer according to the manufacturer's instructions. Sequence data of *X. laevis* genes were collected from public databases. Table 1 shows the names of genes investigated and primer sequences for RT-qPCR.

RNA species of interest were amplified with the Power SYBR Green Master Mix and ABI Prism 7000 sequence detection System (Applied Biosystems) with a specific primer set (Table 1). The PCR protocol consisted of1 cycle of 50°C (2 min) and 95°C (10 min), and then 40 cycles of 95°C (15 s) and 60°C (1 min).The amounts of RNA species were quantified in triplicate using the formula $2^{-\Delta Cq}$, where the endpoint, Cq, was defined as the PCR cycle number that crossed an arbitrarily placed signal threshold. Control reactions without reverse transcriptase were tested for residual genomic DNA, and contamination was evaluated by non-template controls without RNA into

Table 1: Primers and PCR efficiencies.

Gene	Accession Number	Sequence (position)	Slope	Efficiency (%)
<RT-qPCR>				
crebbp	NM_001095168	sense: 5'-TGGAATATGTGAAAAAACTTGGCTAT-3' (4538-4563) antisense: 5'-TCGTCCCCTTCACTTGGTGGGC-3' (4607-4586)	-3.299	100.98
kat2a	XB-GENE-6486305	sense: 5'-GGAGGCGCCGGATTACTAC-3' (2163-2181) antisense: 5'-CAGCCTCTCCGTCATGGTTT-3' (2229-2210)	-3.258	102.75
kat2b	XB-GENE-6488070	sense: 5'-AGACTAATGATCTTGGAATTCAAGCA-3' (1046-1071) antisense: 5'-GTGAAGGGCTTGCAGCATAAG-3' (1126-1106)	-3.354	98.68
ncoa1	XB-GENE-6488444	sense: 5'-TGCGGTGGAGTGATTGAGAA-3' (46-65) antisense: 5'-TTCACGACAAAGAAGAATCCATCTA-3' (122-98)	-3.275	102.01
ncoa2	NM_001087670	sense: 5'-TGTCCAAAATTATCGGCTGTCA-3' (2463-2484) antisense: 5'-CCAGAATTTCATCCAGGTTATCAAA-3' (2562-2538)	-3.215	104.67
ncoa3	NM_001088263	sense: 5'-GTGGCCCGATGCAATAATG-3' (3003-3021) antisense: 5'-TCGTCCAAAGAATTCCTTCCTAAC-3' (3080-3057)	-3.256	102.81
p300	XB-GENE-6487298	sense: 5'-TCATGCAAAGCTTGGGATACTG-3' (3551-3572) antisense: 5'-GGGTATTGTACAGAGTTGCTTTCCA-3' (3642-3618)	-3.365	98.25
hdac1	NM_001088022	sense: 5'-GGATAGACTTGGATGCTTCAATTTG-3' (921-945) antisense: 5'-TCCAGCAACGAGCCACATT-3' (1054-1036)	-3.407	96.57
hdac3	BC070873	sense: 5'-TGGACTGCACCATGCTAAAAAA-3' (444-465) antisense: 5'-GCTCAAGTATGCCAATGACAATG-3' (520-498)	-3.152	107.62
sirt1	NM_001097726	sense: 5'-CCGAGGAGTTACCCAATGGA-3' (339-358) antisense: 5'-AGCCACCAGCAAGATCATCAT-3' (425-405)	-3.289	101.38
akt1	NM_001090409	sense: 5'-TTGCCAGAAGCCAAGTCTTTG-3' (1120-1140) antisense: 5'-TCTGGCCCACCACCTAACC-3' (1175-1157)	-3.011	114.87
prkaa2	NM_001090413	sense: 5'-GCTGCACCGGAAGTTATTTCAG-3' (673-694) antisense: 5'-GATAACCCCGCAGCTCCATA-3' (725-706)	-3.086	110.87
actb	NM_001088953	sense: 5'-GCCCGCATAGAAAGGAGACA-3' (9-28) antisense: 5'-GCGGCAATATCGTCTTCCAT-3' (77-58)	-2.876	122.7
rpl8	NM_001086996	sense: 5'-CAAGGCAAAGAGAAACTGCTG-3' (625-645) antisense: 5'-GCTTACCAATGTGTTGGTGGT-3' (722-702)	-3.496	93.22

Table 1 Contd: Primers and PCR efficiencies.

Gene	Accession Number	Sequence (position)	Slope	Efficiency (%)
<ChIP assay>				
fabp1	AF068301	sense: 5'-TAAAGGGTGTCACCGAGATTG-3' (20-40)	-3.239	103.58
		antisense: 5'-TCTCCCCTGTTGGTGTTTCTA-3' (148-128)		
cdx2	NM_001096486	sense: 5'-CCAGCTCAACTATCGCCAGAA-3' (492-512)	-3.243	103.4
		antisense: 5'-TGGTTTCCTGAGCCATTCG-3' (560-542)		
fxr	NM_001088774	sense: 5'-AAAGGCCAAGGGTTAGCCATAC-3' (394-415)	-3.431	95.64
		antisense: 5'-CAGATGCCTTGTCTCCACACA-3' (474-454)		

the cDNA synthesis. All negative controls had undetermined Cq or Cq > 36. The PCR efficiencies were estimated from cDNA dilution curves and showed linear reaction efficiencies (Table 1). Primer specificity was confirmed by the appearance of a single band on gel electrophoresis and of a single peak on melt curve analysis. Because there was less variation in the Cq values of rpl8 rRNA among the tissues examined (Cq = 24.9 ± 0.7, n = 24) than those of the actb transcript (Cq = 24.1 ± 1.1, n = 24), rpl8 rRNA was used as a reference transcript to standardize each experiment. The amount of a specific transcript was shown relative to that of the rpl8 transcript.

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM) ($n \geq 3$), unless otherwise noted. Differences between groups were analyzed by a one-way analysis of variance, with the Fisher's least significant difference test for multiple comparisons. $p< 0.05$ was considered statistically significant. Statistical analyses were conducted using Microsoft Excel 2003 Data Analysis software (SSRI, Tokyo, Japan).

RESULTS AND DISCUSSION

We first investigated whole cellular changes in the amounts of histone modifications, H4ac, H3K9ac, H3K9me3 and H3K36me3 in intestinal homogenates by Western blotting(Figure 2). However, there were no significant differences in these amounts among the fed, fasted and refed groups, despite most of the genes investigated being down-regulated with 3 weeks of fasting (Tamaoki et al., 2016), suggesting that metabolic rate depression occurs least at the transcriptional level.

In Western blot analyses of histone modifications in brown adipose tissues of thirteen-lined ground squirrels, hibernation reduced the amounts of acetylated histone H3 at lysine 23 (H3K23ac) and phosphorylated histone H3 at serine 10 (H3S10p) by approximately 40% compared with euthermic controls (Morin and Storey, 2006). In the liver and skeletal muscle of freshwater turtles, anoxia treatment decreased the amounts of H3K9ac and H3K23ac by 40 to 60% (Krivoruchko and Storey, 2010). Findings from these studies indicate that the amounts of euchromatin-associated histone modifications are correlated with metabolic rates, including transcriptional and translational activities, during these hypometabolic stress conditions. These findings contrast with our findings, suggesting that there may be some fundamental differences in the regulation of histone modifications between these stress conditions (hibernation and anoxia) and our fasting condition.

Figure 3 shows changes in histone epigenetic marks on the coding region of fabp1 (20–148 bp downstream from 5' end of mRNA), cdx2 492–560 bp) and fxr(394–474bp) genes, which were down-regulated with 3 weeks of fasting and recovered quickly with 1 day of refeeding (Tamaoki et al., 2016).Fasting increased the amounts of H4ac (on fabp1, cdx2 and fxr genes) and H3K36me3 (on fxr gene), which are euchromatin-associated epigenetic marks, and decreased the amounts of H3K9me3 (on fabp1 and cdx2 genes), which is a heterochromatin-associated epigenetic mark. These epigenetic changes returned to the levels of those of the fed frogs with1day of refeeding. Our resultshowed a pattern of epigenetic histone marks similar to those from our previous study (Tamaoki et al., 2016). These observations suggest that histones are epigenetically modified on these genes of the *Xenopus* intestine in response to fasting and refeeding, and confirmed that the amounts of euchromatin-associated histone modifications were correlated inversely with transcript levels (Tamaoki et al., 2016).

The transcript levels of most of the seven HAT genes decreased with fasting, and remained low with refeeding (Figure 4A). The transcript levels of the three HDAC genes decreased with fasting, and recovered quickly to the levels of the fed frogs with refeeding (Figure 4B). Both the akt1 and prkaa2 genes, which are known to be up-regulated in

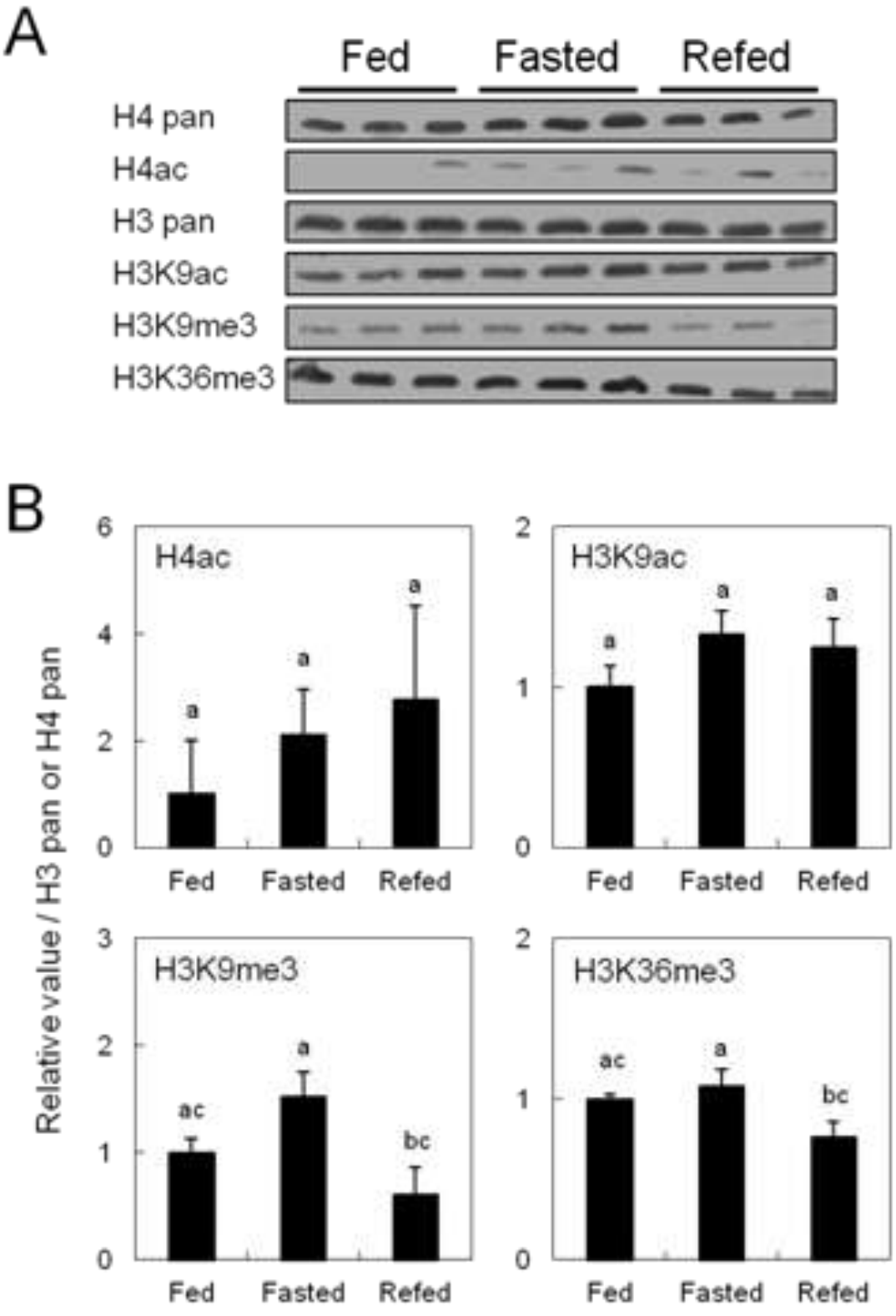

Figure 2. Whole cellular histone modifications in the intestines of the fed, fasted and refed frogs.(*A*) Western blotting analyses of intestinal homogenates (3different samples/each group) with indicated antibodies. (*B*) Quantification of band intensities of Western blotting analyses. Band intensities were analyzed and expressed relative to H4 pan or H3 pan, and values are expressed relative to the mean values of the fed frogs that were set to 1.0. Each value is the mean ± SEM. Different letters denote significantly different means ($p< 0.05$).

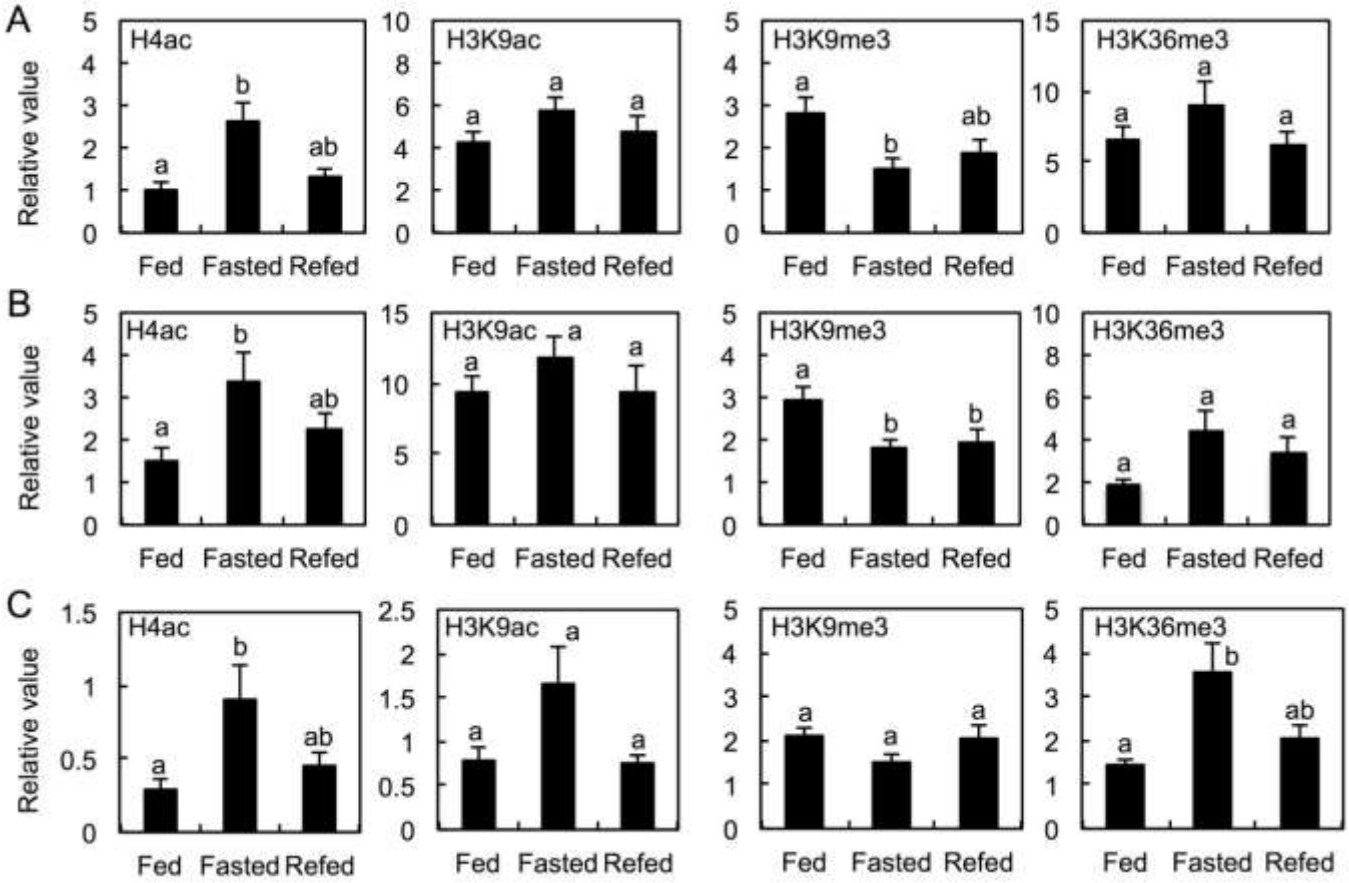

Figure 3. Histone modifications on the coding region of fabp1, cdx2, and fxr genes in the intestines of fed, fasted and refed frogs. Chromatin samples were prepared from the intestines of the frogs that were fed for 22 days (fed), fasted for 22 days (fasted), or fasted for 21 days and then refed for 1 day (refed). Signals of ChIP on fabp1 (*panel A*), cdx2 (*panel B*), and fxr (*panel C*) genes were detected by qPCR following immunoprecipitation with antibodies against H4ac, H3K9ac, H3K9me3 and H3K36me3. Each value is the mean ± SEM. Different letters denote significantly different means ($p < 0.05$).

starved nematodes (Baugh et al., 2009), were not up-regulated in *Xenopus* intestines with 3 weeks of fasting (Figure 4C). The level of akt1 transcript decreased with fasting, and remained reduced with refeeding. The level of prkaa2transcript was not significantly changed with fasting, but decreased with refeeding.

The fluctuations in the amounts of H4ac, H3K36me3 and H3K9me3, on the transcriptionally active genes with fasting and refeeding may in part be explained by the transcriptional regulation of the HDAC genes. However, all of the genes tested in this study, as well as most of the 74 genes tested previously (Tamaoki et al., 2016), showed a similar expression pattern. Therefore, it is likely that the transcript amounts of the HDAC genes may affect most genes under our experimental conditions.

Another mechanism for fluctuations in the acetylated histone levels may be modulation of HAT and/or HDAC activities by post-translational modifications, such as acetylation and phosphorylation (Pham and Lee, 2012), and interactions with other protein factors or metabolic intermediates (Vogelauer et al., 2012). In particular, intermediates of the main metabolic pathways for carbohydrates, amino acids, and fatty acids influence HDAC activities. We detected significant reductions in plasma glucose, triglyceride and free fatty acid concentrations in fasted frogs (Tamaoki et al., 2016), suggesting changes in carbohydrate and lipid usages for energy production, which was probably accompanied by changes in the amount of some of the metabolic intermediates. Sirtuin (SIRT) proteins are an evolutionarily conserved family of class III HDACs, whose activities depend on the presence of the oxidized form of nicotinamide adenine dinucleotide, NAD^+.A recent study showed that SIRT3 concentration and total SIRT activity increased in the skeletal muscle during late hibernation of thirteen-lined ground squirrels (Rouble and Storey, 2015).Ketone bodies are known to be produced in the liver during starvation. One of them, β-hydroxybutyrate, is an inhibitor of class I HDACs (Shimazu et al., 2013). Furthermore, coenzyme A (CoA) derivatives such as acetyl-CoA, as well as NADPH, act as allosteric activators of recombinant HDAC1 and HDAC2, leading to decreases

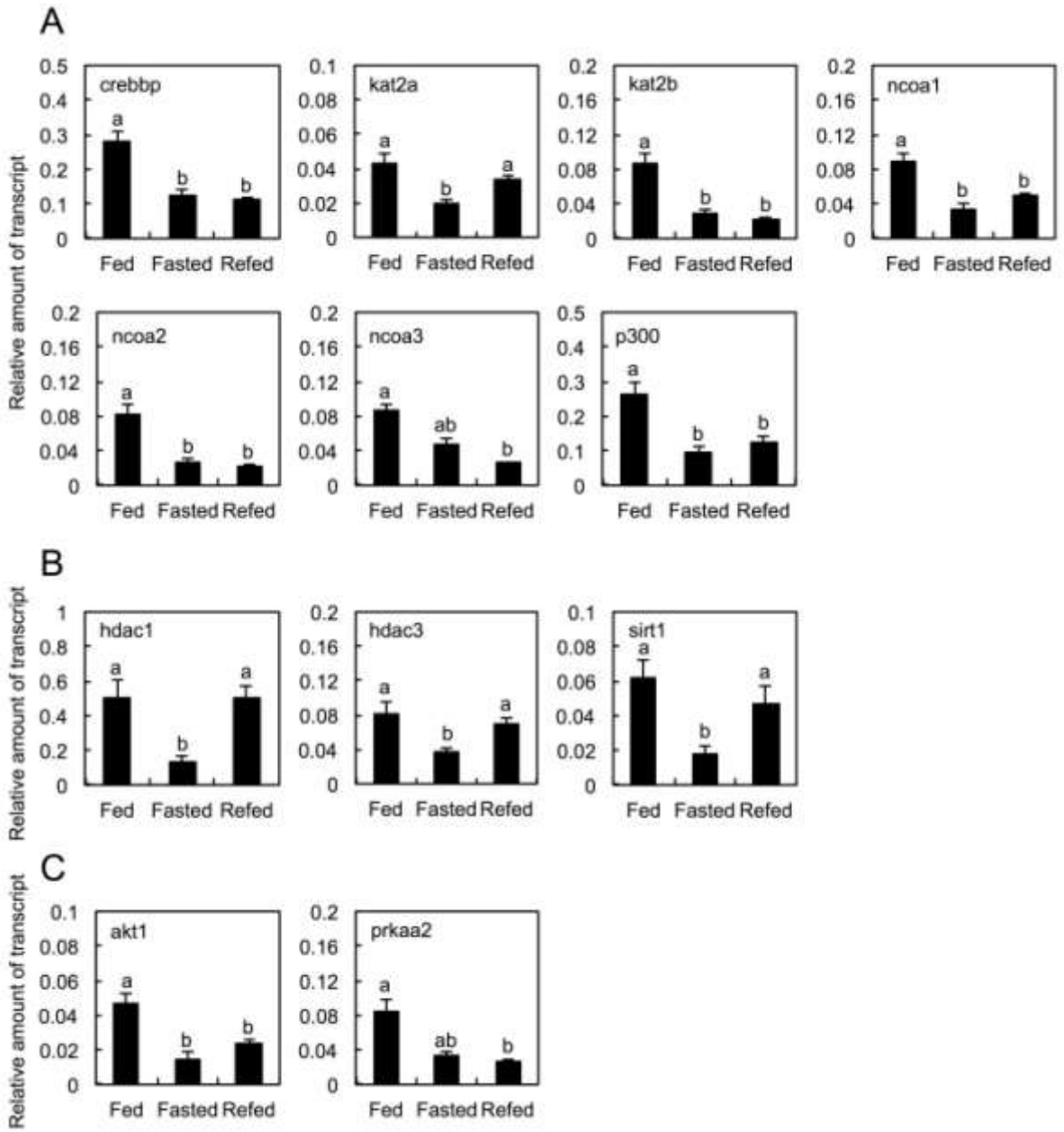

Figure 4. Real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of gene transcripts in the intestines of the fed, fasted and refed frogs. Transcripts of 12 genes were divided into the following three categories: histone acetylation (7 genes; *panel A*), histone deacetylation (3 genes; *panel B*), and others (2 genes; *panel C*). The vertical axis represents the amount of target gene transcript after normalization with rpl8. Each value is the mean ± SEM(n = 8). Different letters denote significantly different means (p< 0.05).

in global levels of acetylated histones H3 and H4 (Vogelauer et al., 2012). Measuring the levels of these metabolic intermediates is necessary to understand the molecular mechanism underlying the fluctuations in the amounts of acetylated histones in the active genes in the *Xenopus* intestines with fasting and refeeding.

Conclusions

In the present study, we clarified that there was no significant difference in the whole cellular levels of histone acetylation among the groups, whereas the amounts of acetylated and methylated histones indicating epigenetic activation marks increased with fasting and decreased with refeedingon three active genes. Fasting for three weeks induced overall suppression of transcript amounts in the *Xenopus* intestines, and one-day refeeding recovered the transcript amounts to the feeding levels. Correlated with these dramatic changes in the transcript amounts, our analysis of global histone acetylation indicates such epigenetic modifications may occur in restricted genomic regions such as active genes. Transcriptional down-regulation of HDAC genes may be one of the possible factors that elevated the levels of acetylated histones on fasting.

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