Biochemical and Biophysical Research Communications 387 (2009) 87–91

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Activity-dependent regulation of HCN1 protein in cortical neurons

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ARTICLE INFO

Article history:
Received 23 June 2009
Available online 27 June 2009

Keywords:
Neuronal activity
Voltage-gated ion channel
Homeostatic plasticity
Surface expression
Cortical neuron

ABSTRACT

Homeostasis of neuronal activity is crucial to neuronal physiology. In dendrites, hyperpolarization-activated cyclic nucleotide-gated channel (HCN) 1 is considered to play critical roles in this process. While electrophysiological studies have demonstrated the dynamic modulation of I_h current mediated by HCN1 proteins, little is known about the underlying molecular and cellular mechanisms. In this study, we utilized cortical cultured neurons and biochemical methods to identify molecular and cellular mechanisms that mediate the physiological regulation of HCN1 channel functions in cortical neurons. Pharmacological manipulations of neuronal activity resulted in changes in the expression level of HCN1. In addition, the surface expression of HCN1 was dynamically regulated by neuronal activity. Both of these changes led to functional modulations of HCN1 channels. Our study suggests that coordinated changes in protein expression and surface expression of HCN1 serve as the key regulatory mechanisms controlling the function of endogenous HCN1 protein in cortical neurons.

The ability to maintain stable overall activity in dynamically changing environments is crucial to the normal physiology of neurons. Accumulating evidence suggests that the intrinsic excitability of neurons is regulated by such homeostatic plasticity [1]. The intrinsic excitability of neurons is shaped by a wide variety of voltage-gated ion channels expressed in the soma and dendrites. Among them, hyperpolarization-activated cyclic nucleotide-gated channels (HCNs) play critical roles in the intrinsic excitability of dendrites [2,3].

In the HCN family, HCN1 is thought to be responsible for the regulation of dendritic integration at the distal excitatory synapses in the central nervous system [4]. In addition, HCN1 has also been shown to play important roles in spatial working memory [5,6], motor learning [7] and anesthesia [8]. These data demonstrate the importance of HCN1 and its regulation in the expression of higher brain functions.

While electrophysiological studies have revealed the modulation of I_h current mediated by HCN1 proteins in both *in vitro* and *in vivo* models [4,9–12], little is known about the underlying molecular and cellular mechanisms. In addition, most studies have focused on the CA1 region of the hippocampus, while knowledge of such regulation in the cerebral cortex is very limited. In this study, we utilized mouse cortical cultured neurons and biochemical methods to identify molecular and cellular mechanisms that mediate the neuronal activity-dependent regulation of HCN1 channel function in neurons. The application of pharmacological and biochemical techniques allowed us to monitor molecular changes in endogenous HCN1 proteins upon changes in neuronal network activity. Our results suggest that not only the protein expression level but also the surface expression of HCN1 is regulated by neuronal activity. This newly revealed physiological regulation of HCN1 may play crucial roles in the expression of homeostatic plasticity in neurons.

Materials and methods

Materials. The reagents for the neuronal cultures were purchased from Invitrogen (Carlsbad, CA, USA); all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise stated. The anti-HCN1 monoclonal antibody N70/28 was developed by and obtained from the University of California at Davis/NINDS/NIMH NeuroMab Facility, supported by NIH Grant U24NS050606 and maintained by the Section of Neurobiology, Physiology and Behavior, College of Biological Sciences, University of California, Davis, CA 95616.

Neuronal cultures. High-density primary culture cortical neurons were prepared from E17 mice as described previously [13]. Briefly, cortices from E17 mice (ICR, SLC, Japan) were dissected and digested with 0.5% papain in Earle's Balanced Salt Solution for 15 min at 37 °C. The cells were plated on poly D-lysine (0.1 mg/ml)-coated tissue culture dishes at a density of 5×10^6 cells/60-mm dish and were maintained in Glia-Conditioned Feeding Medium (5% horse serum, 2 mM GlutaMAX, 100 U/ml penicillin and 100 μg/ml streptomycin in Eagle's Minimum Essential Medium). The neurons were maintained at 37 °C in a humidified

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doi:10.1016/j.bbrc.2009.06.127

incubator with 95% air and 5% CO_2, and were used after 14–21 days of culture. All procedures relating to the care and treatment of the animals were approved by the Animal Resource Committee of the School of Medicine, Keio University.

Calcium imaging. Neurons were loaded with 1 μM Fluo-5F AM (Invitrogen, Carlsbad, CA, USA) in artificial cerebrospinal fluid (aCSF) containing 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM glucose for 30 min at 37 °C. Calcium imaging was performed at RT (20–25°C) using a FV1000 confocal microscope (Olympus, Tokyo, Japan). Time-lapse images were acquired every 1.1 seconds up to 330 s. The numbers of all detected neurons were counted for each field of view in a *t*-stack image. The fluorescence change over time is defined as $\Delta F/F = (F - F_{basal})/F_{basal}$, where F is the fluorescence at any time point and F_{basal} is the baseline fluorescence averaged across the whole movie for each cell, as described previously [14]. Neurons exhibiting at least one transient during the observation period were defined as "active" and the others were defined as "inactive". Numbers of active neurons and numbers of the transients of those neurons were counted manually.

Electrophysiology. Electrophysiological recordings were performed on high-density cortical cultured neurons in aCSF at RT using MultiClamp 700B (Molecular Devices, Sunnyvale, CA, USA). A glass pipette (6–12 MΩ) was filled with internal solution containing 135 mM $KMeSO_4$, 5 mM NaCl, 10 mM KCl, 2.5 mM Mg-ATP, 0.3 mM Na-GTP and 10 mM HEPES at pH 7.3. After achieving a whole-cell configuration, cells were held in the current-clamp mode to record neuronal activity. For voltage sag measurements, 1 second-long hyperpolarizing current (−100 pA) was injected into neurons and the sag ratio was calculated as steady-state/trough voltage, as reported previously [15]. For comparison of sag ratio, data from neurons exhibiting a clearly recognizable voltage sag (>1 mV) were used (control: 25/32 cells, TTX: 23/27 cells).

Pharmacology and sample preparation. For the recovery experiments, neurons were treated with 1 μM tetrodotoxin (TTX) for 24 h and washed with culture medium twice, then incubated in regular medium for an additional 24 h. For the controls, cells were either incubated without TTX for 48 h or were incubated with TTX for only the final 24 h. For the time course experiments, cells were incubated with TTX for 0 min, 5 min, 30 min, 2 h or overnight. For the glycosidase assay, cells were either incubated without TTX for 24 h or were incubated with TTX for 24 h. Following TTX treatment, cells were lysed in modified RIPA buffer (1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 50 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, 2 mM EGTA, 50 mM NaF, 10 mM sodium pyrophosphate [pH 7.4] and complete protease inhibitor cocktail [Calbiochem, Darmstadt, Germany]), and the soluble fraction was used after centrifugation (16100*g*, 15 min, 4 °C).

SDS–PAGE and immunoblotting. Protein samples were resolved by SDS–PAGE on 7.5% polyacrylamide gels, transferred to PVDF membranes, and processed for immunoblotting. Membranes were probed overnight at 4 °C with anti-HCN1 or anti-β-actin antibodies. All immunoblots were visualized using SuperSignal substrate (Pierce, Rockford, IL, USA), and the X-ray films were scanned using a GS-800 densitometer (Bio-Rad Laboratories, Hercules, CA, USA) followed by quantification using Quantity One for band intensities (pixel intensity x area) and Image J for intensity profiles.

Glycosidase assay. Glycosidase assays were performed essentially according to the manufacturer's protocol. Briefly, lysates were incubated with Glycoprotein Denaturing Buffer for 5 min at 100 °C and the enzyme reactions were performed using PNGase F, Endo H (New England Biolabs, Ipswich, MA, USA) or water for 1 h at 37°C. The SDS samples were prepared and processed for immunoblotting as described above.

BS^3 surface crosslinking. Bis[sulfosuccinimidyl]suberate (BS^3) surface crosslinking was performed as described previously [16]. Briefly, cells were washed with aCSF and incubated with 1.0 mg/ml BS^3 (Pierce) in aCSF for 30 min at 4 °C to label surface proteins with BS^3. Remaining reactive BS^3 was quenched by incubating the cells with Tris-buffered saline three times each for 5 min at 4 °C.

Statistical analysis. All results are presented as means ± SEM for the indicated number of experiments. Statistical significances were determined using one-way ANOVA followed by the Fisher's *post hoc* test. For the sag ratio comparison, the Mann–Whitney test was employed.

Results

Manipulation of neuronal network activity

To examine how neuronal activity regulates cortical HCN1 protein at the cellular and molecular level, we utilized dissociated cortical cultured neurons and investigated the effect of TTX, as previously described [17,18]. As a characterization of the system, electrophysiological properties of neurons were examined. Neurons were randomly selected and patch-clamped in the current-clamp mode to monitor their activity. As seen in Fig. 1A, neurons in control conditions received heavy synaptic inputs and fired action potentials as a result. In contrast, neurons treated with TTX overnight showed no sign of action potentials or any synaptic inputs. When these neurons were returned to normal media and allowed to recover for an additional day, neuronal activity and network activity recovered to control levels. To further support these observations and to investigate neuronal population activity, calcium imaging of neuronal networks was performed (Fig. 1B–D). This approach allows the investigation of neuronal activity by detecting membrane depolarization-dependent influx of calcium ions into neurons [14]. The proportion of active neurons and the average number of calcium transients in all neurons were analyzed. The average number of the calcium transients was calculated by dividing the total number of calcium transients by the number of total neurons (i.e. active neurons and inactive neurons) in each condition. In agreement with the electrophysiological data, the population of active neurons as well as the number of calcium transients significantly decreased under TTX and recovered to original levels upon withdrawal of TTX (percentage active neurons and average number of transients; control: 69.7 ± 0.7%, 4.5 ± 0.9 transients, $n = 9$; TTX: 1.5 ± 1.0%, 0.0 ± 0.0 transients, $n = 10$; recovery: 69.7 ± 0.7%, 4.5 ± 0.9 transients, $n = 5$; $p < 0.01$, ANOVA). These data demonstrate that neuronal network activity can be reversibly manipulated pharmacologically in our experimental system.

Expression of HCN1 is regulated by neuronal activity

Having established the experimental model, the protein expression levels of HCN1 under these pharmacological manipulations were compared. To standardize the total protein levels, the HCN1 band intensity was normalized to that of β-actin (Fig. 2A and B). The suppression of neuronal activity with TTX led to a significant decrease in HCN1 protein expression to 72.4 ± 6.0% ($p < 0.02$, $n = 6$) relative to that under the control condition, which then recovered to original level after the termination of TTX treatment (103.9 ± 6.9%, $p = 0.7$, $n = 6$). These lines of evidence suggest that the total protein expression level of HCN1 is directly regulated by neuronal activity in cortical neurons.

Activity-dependent regulation of HCN1 surface expression

In order to determine how fast HCN1 is regulated by neuronal activity, we performed time-course experiments. While a significant reduction of HCN1 protein became evident only after 2 h of

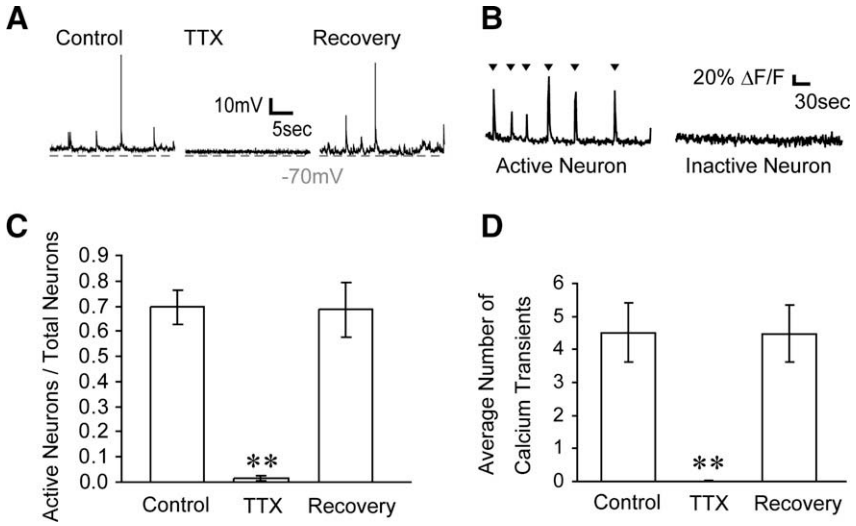

Fig. 1. Pharmacological manipulations of neuronal network activity. (A) Representative voltage traces of neurons in each condition. Neurons were untreated (control), treated with TTX for 24 h (TTX) or allowed to recover for 24 h in a normal medium after 24 h TTX treatment (recovery) and their electrical activities were assessed by current-clamp recordings. (B) Representative calcium dynamics of "active" and "inactive" neurons. Neurons were loaded with Fluo-5F AM and their calcium dynamics were recorded by time-lapse imaging. Arrowheads indicate calcium transients. (C,D) Summary of calcium dynamics in each condition. Proportion of active neurons (C) and average number of calcium transients (D) are shown. $^{**}p < 0.01$ ($n = 9$ for control, $n = 10$ for TTX, $n = 5$ for recovery, one-way ANOVA with Fisher's *post hoc* test).

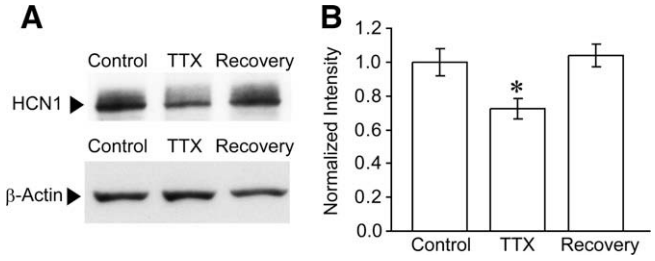

Fig. 2. Activity-dependent regulation of endogenous HCN1 in cortical cultured neurons. (A) Representative western blots of HCN1 and β-actin upon treatment of cortical neurons with TTX and following recovery. (B) Quantification of Western blots in A. $^{*}p < 0.05$ ($n = 6$ for each group, one-way ANOVA with Fisher's *post hoc* test).

TTX treatment, gradual changes in the HCN1 band patterns were clearly observed (Fig. 3A). To clearly visualize this shift, changes in the electrophoresis mobility patterns were analyzed by measuring the intensity profile of the band toward a lower molecular weight [19] (Fig. 3B). Interestingly, the initial broad band of HCN1 shrank toward a lower molecular weight as a result of a decrease in higher molecular weight species. As HCN1 is glycosylated *in vitro* and *in vivo* [20,21], which generally broadens the band toward a higher molecular weight, we performed an *in vitro* glycosidase reaction. Higher molecular weight areas for HCN1 bands from both control and TTX treated samples shifted to a lower molecular weight with PNGase F but not with Endo H (Fig. 3C). Importantly, differences in migration patterns observed between control and TTX treated neurons were abolished upon PNGase treatment. These data demonstrate that HCN1 is basally modified by Endo H-insensitive *N*-glycosylation in cortical neurons and suggest that the suppression of neuronal activity leads to a time-dependent decrease in *N*-glycosylation.

These changes in the glycosylation patterns suggest that the surface expression of HCN1 is also regulated by neuronal activity. To test this possibility, we utilized BS^3, a membrane-impermeable divalent crosslinker that selectively crosslinks proteins expressed on the plasma membrane. As reported previously, crosslinked proteins migrate very slowly on SDS–PAGE and are clearly distinguishable from non-crosslinked proteins [16] (Fig. 3D). Immunoblotting the same blot with anti-β-actin antibody did not reveal any high molecular weight surface crosslinked proteins (data not shown), confirming the specificity of this method for specifically labeling proteins expressed on the plasma membrane. To compare the surface expression level, the band intensity of crosslinked surface HCN1 expressed on the plasma membrane was divided by that of the remaining non-crosslinked HCN1 (Fig. 3E). Treatment of the cells with TTX dramatically decreased the surface crosslinked population to 54.3 ± 10.6% ($p < 0.05$, $n = 3$), as compared to control conditions. In contrast, the examination of other dendritic membrane proteins, such as Kv4.2, did not reveal any change in surface expression (data not shown), demonstrating the specificity of this change induced by neuronal activity. Furthermore, the surface expression of HCN1 reverted to 94.9 ± 9.9% ($p = 0.73$, $n = 3$) of the control level with the recovery of neuronal activity. Together with the changes in the glycosylation patterns, these data demonstrate that not only the total protein expression level but also the surface expression of HCN1 is specifically regulated by the neuronal network activity.

Effect of neuronal activity on HCN1 function

The biochemical data described above suggest that the function of HCN1 is regulated by neuronal activity. To confirm this possibility, whole-cell patch-clamp recordings were performed and voltage sag was measured. Voltage recordings in cortical neurons from HCN1 knockout mice suggest that the voltage sag is mainly attributed to HCN1 in these neurons [22]. As reported previously in dissociated hippocampal neurons, there was a large variability in the sag among randomly sampled cortical neurons [15]. As predicted, when neurons were treated with TTX overnight, a clear tendency for a decrease in the voltage sag was observed. To compare the function of HCN1 using voltage sag as an indicator, sag ratios of neurons exhibiting significant voltage sag were pooled and compared. As shown in Fig. 4, sag ratio was significantly reduced upon TTX treatment (control: median = 0.898, interquartile range = 0.778, 0.965, $n = 25$; TTX: median = 0.964, interquartile range = 0.903, 0.973, $n = 23$; $p < 0.02$,

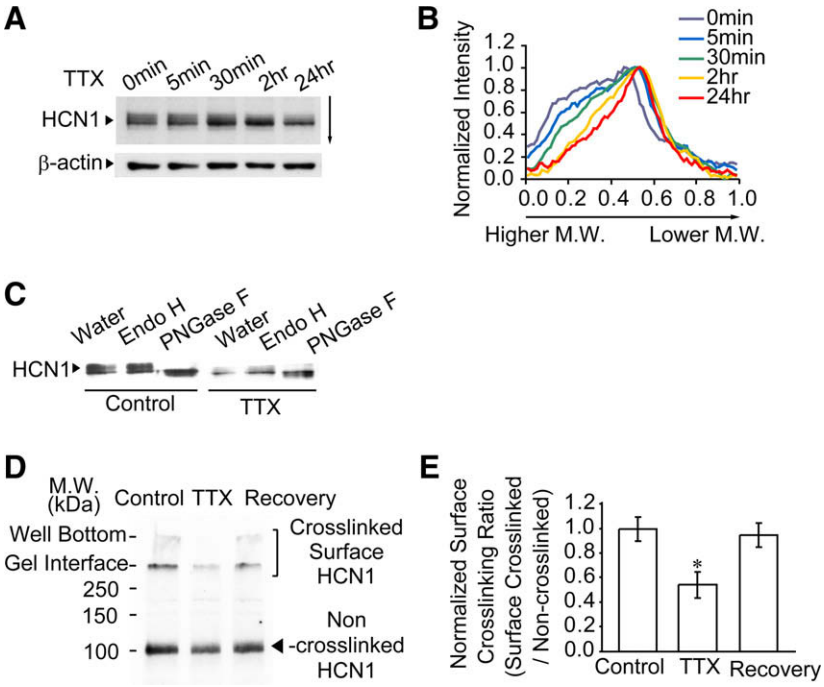

Fig. 3. Activity-dependent regulation of HCN1 surface expression. (A) Representative Western blots of HCN1 and β-actin collected from cortical neurons treated with TTX for the indicated periods. The arrow indicates the direction of the intensity profile in B. (B) Intensity profiles of the bands shown in A. The arrow indicates the direction of the intensity profile shown in A. (C) *In vitro* glycosidase reaction on endogenous HCN1 proteins in cortical cultured neurons. Bands of HCN1 from both control and TTX treated samples shifted to a lower molecular weight with PNGase F but not with Endo H. These experiments were performed four times and essentially the same results were obtained. (D) Representative western blot of BS^3 treated cortical neuron lysate probed for HCN1. Surface HCN1 proteins crosslinked with BS^3 as well as non-crosslinked proteins are indicated. (E) Quantification of western blot shown in D. $^*p < 0.05$ ($n = 3$ for each group, one-way ANOVA with Fisher's *post hoc* test).

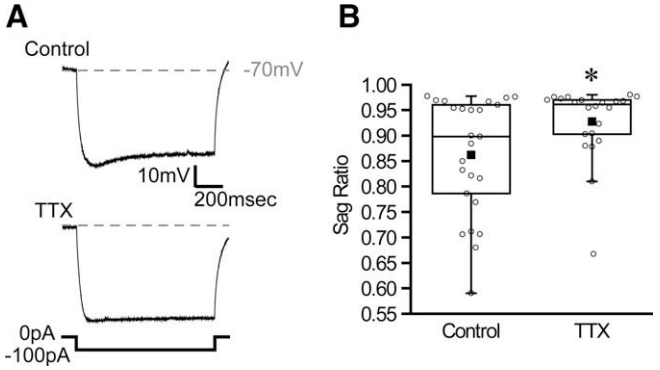

Fig. 4. Effect of neuronal activity on voltage sag. High density cortical cultured neurons from untreated and TTX treated dishes were patch-clamped and the voltage sags were measured. (A) Representative traces of voltage sag in control and TTX-treated neurons. (B) Summary of voltage sag measurements. The panel shows the box chart of all the data collected (open circles) with the mean value shown in filled squares. The box shows 25th, 50th and 75th percentile points and the whisker lines show the distribution of data without outliers (lower point: 25th Percentile – (1.5 * Interquartile Range), upper point: 75th Percentile + (1.5 * Interquartile Range)). $^*p < 0.05$ ($n = 25$ for control, $n = 23$ for TTX, Mann–Whitney test).

Mann–Whitney test). This result suggests that biochemical changes in HCN1 induced by neuronal activity indeed result in functional modification of HCN1 in cortical neurons.

Discussion

Although our knowledge of electrophysiological properties has advanced, little is known about the activity-dependent regulation of endogenous HCN1 proteins at the molecular and cellular levels. Moreover, while the concept of functional regulation of channels through dynamic changes in trafficking has been well established for many other neuronal channels, very little is known about such physiological regulation of endogenous HCN1 channels in cortical neurons. To our knowledge, this is the first study to report that both total protein expression and surface expression are the key mechanisms regulating HCN1 during changing neuronal activity in cortical neurons.

At least four major mechanisms that regulate the function of HCN1 exist: total protein expression, surface expression, channel properties and tetramer formation, all of which can be modulated by posttranslational modifications and/or protein-protein interactions [23,24]. Our results indicate that surface expression, together with the total protein expression level, is physiologically regulated by the activity of the neuronal network. In agreement with previously reported electrophysiological findings, both the total protein expression and the surface expression of HCN1 were decreased when the neuronal network was suppressed under our experimental conditions. Furthermore, the time-dependent change in endo-H-insensitive *N*-glycosylation implies that HCN1 trafficking may stop at the endoplasmic reticulum or Golgi apparatus when neuronal activity decreases. These changes in HCN1 protein expression would modulate the intrinsic excitability of neurons, controlling changes in network activity and thereby contributing to the homeostatic plasticity.

Previous studies have reached somewhat contradictory conclusions about the regulation of HCN1 channels. Increased neuronal activity in the presence of high extracellular K^+ reportedly induced an increase in total HCN1 protein expression in hippocampal slice cultures, in agreement with our study [9]. However, *in vivo* seizure-like events induced by kainic acid decreased the total HCN1 protein expression in the hippocampus [21]. In another study using hippocampal slice cultures, the suppression of neuronal activity by treatment with TTX from DIV14 for 48 h did not significantly decrease

HCN1 protein expression [25]. The reasons for the apparent discrepancies between our data and these previous studies are not clear, but might include differences in brain region and stage of the samples studied. Further investigations are needed to examine this potential region-specific regulation of HCN1 proteins. Considering the previously reported importance of HCN1 in the expression of higher brain functions such as working memory in the forebrain [5,6], our data from cortical neurons might be of particular interest in elucidating the cellular mechanisms of cortex-dependent brain functions.

Acknowledgments

This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan, Keio University Research Grants for Life Science and Medicine, The Ichiro Kanehara Foundation and Global COE Program "Center for Human Metabolomic Systems Biology" from MEXT, Japan. We would like to thank Manae Imamura for excellent technical help, Mitsuko Shiota for administrative work, and members of the laboratory for their comments.

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