Identification of the Downstream Targets of SIM1 and ARNT2, a Pair of Transcription Factors Essential for Neuroendocrine Cell Differentiation*

SIM1 and ARNT2 are two basic helix-loop-helix/PAS (Per-Arnt-Sim) transcription factors that control the differentiation of neuroendocrine lineages in the mouse hypothalamus. Heterozygous Sim1 mice also develop early onset obesity, possibly due to hypodevelopment of the hypothalamus. Although SIM1 and ARNT2 form heterodimers to direct the same molecular pathway, knowledge of this pathway is limited. To facilitate the identification of their downstream genes, we combined an inducible gene expression system in a neuronal cell line with microarray analysis to screen for their transcriptional targets. This method identified 268 potential target genes of SIM1/ARNT2 that displayed >1.7-fold induced expression. 15 of these genes were subjected to Northern analysis, and a high percentage of them were confirmed to be up-regulated. In vivo, several of these genes showed neuroendocrine hypothalamic expression correlating with that of Sim1. Furthermore, we found that expression of two of these potential targets, the Jak2 and thyroid hormone receptor β2 genes, was lost in the neuroendocrine hypothalamic of the Sim1 mutant. The expression and predicted functions of many of these genes provide new insight into both the Sim1/Arnt2 action in neuroendocrine hypothalamic development and the molecular basis for the Sim1 haplo-insufficient obesity phenotype.

The neuroendocrine hypothalamus mediates homeostasis by regulating peptidergic hormone secretion of the pituitary. Discrete hypothalamic secretory neurons mediate this function. These neurons include the oxytocin (OT)-, vasopressin (VP)-, corticotropin-releasing hormone (CRH)-, thyrotropin-releasing hormone (TRH)-, and somatostatin-producing neurons. OT and VP neurons project to the posterior pituitary, where they release hormones directly into the bloodstream, whereas the CRH, TRH, and somatostatin neurons project to the median eminence, which in turn carries their secreted hormones to the anterior pituitary to modulate pituitary secretion (1). Despite extensive studies of the physiological functions of these hormones, the molecular pathways directing their expression in specific cell lineages are less well known.

Analyses of Sim1 and Arnt2 mutant mice have demonstrated that these transcription factors are essential for the terminal differentiation of the aforementioned neurons (2–5). In the absence of either gene, the precursors of these neurons are born normally, but fail to form the anatomical neuroendocrine centers, i.e. the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) in the anterior hypothalamus, and do not produce any of the hormones (2–5). The collective loss of these neuroendocrine hormones may cause the observed perinatal lethality of the Sim1 and Arnt2 mutants (2–5). Intriguingly, heterozygous Sim1 mice develop early onset obesity, proposed to be due to hypodevelopment of the neuroendocrine hypothalamus (6). A balanced chromosomal translocation disrupting Sim1 (7) and a haploid interstitial deletion of chromosome 6 encompassing SIM1 (8) have also been shown to be associated with profound obesity in humans.

Sim1 and Arnt2 are homologs of Drosophila sim and tango, respectively (9–14). These genes belong to the family of basic-helix-loop-helix (bHLH)/PAS (Per-Arnt-Sim) domain-containing proteins, many of which are important regulators of development and physiology (14). DNA binding assays in vitro have demonstrated that SIM1 and ARNT2 form heterodimers and bind the core sequence TACGTC, named central nervous system midline enhancer (CME) (3, 12, 14–16). The CME was originally identified in the enhancer regions of sim/tango downstream genes (13, 15). Multimerized CME can mediate sim/tango-dependent central nervous system midline expression in the fly (11, 13–16). When linked to a minimal adenovirus major late promoter-driven reporter gene, CME can also mediate Sim1/Arnt2-dependent transcriptional activation of the reporter in cultured mammalian cells, albeit weakly (17). Deletion analyses of Sim1 and Arnt2 demonstrate that their basic domains are required for CME recognition, their bHLH/PAS domains for heterodimerization, and their C termini for transcriptional regulation (9, 12, 17, 18). However, when the Sim1 C terminus is fused to the Gal4 DNA-binding domain and tested in a different cell line using a Gal4-thymidine kinase promoter-driven reporter, it acts as a repressor (9). These results suggest that SIM1 can act as a repressor or an activator depending on the context of the reporter assay.

Brn2, a POU domain-encoding gene, is a downstream target of Sim1/Arnt2 in vivo (2–5). In both Sim1 and Arnt2 mutants, Brn2 expression in the prospective neuroendocrine cells is lost.

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The on-line version of this article (available at http://www.jbc.org) contains Supplemental Fig. 1.

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‡ The abbreviations used are: OT, oxytocin; VP, vasopressin; CRH, corticotropin-releasing hormone; TRH, thyrotropin-releasing hormone; bHLH, basic helix-loop-helix; PVN, paraventricular nucleus; SON, supraoptic nucleus; CME, central nervous system midline enhancer; IRES, internal ribosomal entry site; Dox, doxycycline; MC3R, melanocortin-3 receptor; TRβ2, thyroid hormone receptor β2; IL-6Ra, interleukin-6 receptor α; ISH, in situ hybridization; E18.5, embryonic day 18.5; NLOT, nucleus of the lateral olfactory tract; AVN, anteroventral nucleus; rTATA, reverse tetracycline-controlled transactivator; tTS, tetracycline transcriptional silencer; bHLH, basic helix-loop-helix.

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Furthermore, Brn2 mutant mice have a selective defect in CRH-, VP-, and OT-expressing neurons (19–21), which is a subset of the Sim1 and Arnt2 mutant defects. Brn2 has also been shown to bind to the CRH promoter and to activate its transcription (22, 23). The genes employed by SIM1/ARNT2 to specify the other neuroendocrine hormone gene expression in distinct cell types remain unexplored.

To study the molecular pathways by which Sim1 and Arnt2 control the development of the hypothalamic secretory neurons and mediate energy homeostasis, we combined an inducible gene expression system with microarray analysis to screen for their downstream targets. Below, we describe the genes identified by this screening and the resulting implications for the Sim1/Arnt2-operated molecular pathway.

EXPERIMENTAL PROCEDURES

**Plasmids**—The SIM1 N-terminal BHLH/PAS domain (1044 bp from ATG to an internal EcoRv, referred to as SIMN) was fused in-frame with Gal4 or VP16 activation domains. These fusion forms of Sim1 and full-length Sim1 cDNAs were cloned into a pIRES vector (Clontech) with Gal4 or VP16 cDNA inserted 3′ to the internal ribosomal entry site (IRES) to make pIRES-Sim1-Gal4VP16-Arnt2, pIRES-Sim1-Gal4VP16-Arnt2, and pSIMN-Gal4-IRES-Arnt2 cassettes. These cassettes were cloned into the pTRE2hyg vector (Clontech). In addition, Sim1, Arnt2, and SimN-VP16 were individually cloned into pTRE2hyg to make pTRE-Sim1, pTRE-Arnt2, and pTRE-Sim-VP16, respectively. pTet-On and pTet-tTS (Clontech) were used for expression of the tetracycline-regulated activator rtTA and repressor TTS, respectively, to render doxycycline (Dox)-dependent regulation of pTRE-driven expression of cloned cDNAs. The CME-driven luciferase reporters pML/6C-WT and pML/6C-AM were gifts from Dr. J. Pelletier (17). 50 ng of pSv-B-gal (Invitrogen) was included in all transfections, and the β-galactosidase activity was measured (LacZ assay kit, Promega) for normalization.

**Cell Line**—Neuro-2a cells (American Type Culture Collection, Manassas, VA) were cultured in Eagle’s minimal essential medium (Vitacell, American Type Culture Collection) and 10% bovine serum. FuGENE 6 reagent (Roche Applied Science) was used for DNA transfection. For transient transfections, each plasmid was used at 250 ng in a final 1 μg of total DNA for each well of a 6-well dish (Falcon). The plasmids used for each transfection are indicated in the figures. For a stable cell line, Neuro-2a cells were transfected with 10 μg of pTet-On and selected with 200 μg/ml G418 (Sigma) to obtain individual clones. Selected colonies were propagated and transfected with pTRE2hyg-Luc (Clontech) to test their Dox (1.5 μM/ml; Clontech) responsiveness by assaying luciferase activity. The clone with the lowest background was transfected with 2 μg of pTRE-SN-VP16 and 10 μg of pTet-tTS and selected with 150 μg/ml hygromycin (Roche Applied Science) to obtain secondary clones. Individual clones were then tested for their Dox-regulated SIMN-VP16/ARNT2 activity by assaying for pML/6C-WT reporter activity under mock and Dox treatment conditions. Clone 37 was chosen for microarray study. Luciferase activities were measured by luciferin (Sigma) light emission using Monolight 2000 (Analytical Luminescence Laboratory).

**Microarray Hybridization and Data Analysis**—Total RNA was isolated using TRIzol reagent (Invitrogen), followed by the Qiagen RNaseasy method. Microarray hybridization using the MG-U74v2A gene chip was performed using a service provided by Neurologic Functional Genomics. Triple-trans hydridizations with independently synthesized probes were conducted using the same batch of RNA isolated from the untreated and Dox-treated clone 37 cell lines and the parental cell line. Hybridization signals were normalized by Affymetrix Suite software. The data sets were filtered by absent and present cells using the Affymetrix Datamining tool, i.e. genes that displayed inconsistent signals within the same oligonucleotide probe set are excluded. Based on the general background of the data, genes displayed signals >30 arbitrary units under both mock and Dox treatment conditions (after normalization) were excluded for further analyses. The remaining genes were subjected to Student’s t test, with p ≤ 0.05 considered significant. We arbitrarily chose a 1.7-fold increase as a cutoff threshold for selecting genes for microarray analysis based on Northern confirmation rate of genes displaying various fold inductions.

**Northern and Western Analyses**—10 μg of total RNA was resolved on 1% agarose gels and transferred to GeneScreen membrane (PerkinElmer Life Sciences) for Northern hybridization. For the melanocorticot 3 receptor (MC3R) and Tbr1 genes, fragments were amplified by reverse transcription-PCR using total RNA isolated from newborn mouse brain. The primers used were 5′-ggaactgctgctt-3′ and 5′-catggcaagatg-3′ for the MC3R gene and 5′-gaacactctggagaa-3′ and 5′-aaggtgcgggtgc-3′ for Tbr1. For other genes, IMAGE clones were obtained from American Type Culture Collection and ResGen: Tlx2 (clone 935644), Jnk2 (clone 1391934), Mtpn (clone 5579590), thyroid hormone receptor β2 (TRβ2, clone 1606024), Chreme (clone 5127017), Grin1 (clone #11-10-U1), Naca (clone 2136152), and interleukin-6 receptor α (IL-6Rα, clone 1463277). Each clone was sequenced to confirm authenticity. The glyceraldehyde-3-phosphate dehydrogenase probe is a PCR fragment obtained from Clontech. The DNA fragment of each gene was labeled with α-32P[αCTP] by random priming (Stratagene) and used for hybridization according to the protocol provided by PerkinElmer Life Sciences for using the GeneScreen membrane.

Cell lysates were resolved on a 4–15% gradient gel (Bio-Rad); transferred to a Hybond membrane (Amersham Biosciences); and probed with antibodies against the VP16 activation domain (Clontech), ARNT2 (Santa Cruz Biotechnology), and γ-tubulin (Sigma). Horseradish peroxidase-conjugated secondary antibodies (Sigma) linked to the chemiluminescence reaction (Amersham Biosciences) were used to visualize these proteins.

**In Situ Hybridization (ISH)**—The brains of CD1 mice at embryonic day 18.5 (E18.5; vaginal plug date is designated as embryonic day 0.5) were snap-frozen in OCT compound and cryosectioned at 20-μm thickness. In Fig. 1A, Brn2 heterozygotes in a BL6 backcrossed background were mated to obtain E18.5 brains from Sim1 mutant and wild-type siblings for ISH. Digoxigenin-labeled sense and antisense probes of the genes specified in the figures were synthesized using SP6, T7, or T3 polymerase and used at 1 μg/ml for hybridization of the brain sections according to Scharzen-Wuenners and Gerfin-Moser (24).

**RESULTS**

**Inducible Expression of SIM1 and ARNT2 in the Neuro-2a Cell Line**—Overexpression of transcription factors should lead to changes in the expression levels of their downstream target genes. To achieve controlled SIM1/ARNT2 overexpression, we chose to use the Tet-On inducible system (see “Experimental Procedures” for details). In this system, pTet-On and pTet-tTS (Fig. 1A) are used to confer tetracycline-dependent regulation of pTRE (tetracycline-responsive element)-driven genes. In this case, Sim1 and Arnt2 (Fig. 1A; diagrammed in Fig. 3A).

We reasoned to implement this system in a neuronal cell line, as we are interested in the function of Sim1/Arnt2 in the hypothalamus. Because the SIM1 C terminus has been reported to repress or activate transcription in different contexts (9, 17), we surveyed the activity of SIM1/ARNT2 in various neuronal cell lines using the CME-driven luciferase reporter (pML/6C-WT) assay devised by Moffett and Pelletier (17). SIM1 and ARNT2 together activated this reporter expression in NB41A3, N1E-115, and Neuro-2a cells in a CME-dependent manner (data not shown). The Neuro-2a cells were chosen for further study due to their homogeneous morphology and high transfection efficiency.

Under the various Dox treatment conditions tested, we observed an optimal ~4-fold activation of the pML/6C-WT reporter by SIM1 and ARNT2 in Neuro-2a cells (Fig. 1B), regardless of whether they were transfected from a single plasmid linked by the IRES or from separate plasmids (data not shown). Concerned that their activity might be too low to activate endogenous genes for our assay, we constructed a potent SIM1 fusion activator. Sim1 hybrid constructs with the SIM1 C terminus replaced by the Gal4 and VP16 (SIMN-VP16) activation domains were cloned into the pTRE2hyg vectors pTRE-SN-Gal4 and pTRE-SN-VP16, respectively (Fig. 1A). Arnt2 was placed downstream of the IRES located 3′ to these Sim1 variants for coexpression (Fig. 1A). Plasmids carrying these Sim1 variants as well as Arnt2 were transfected into Neuro-2a cells to compare their Dox-regulated proficiency in activating the pML/6C-WT reporter. Upon Dox treatment, pTRE-SN-VP16 conferred a 27-fold activation of the reporter compared with the...
12- and 4-fold reporter activation rendered by pTRE-SN-Gal4 and pTRE-SF, respectively (Fig. 1B). SIMN-VP16 appears to function with similar specificity as SIM1, as it also required ARNT2 and wild-type CME sites for reporter gene activation (Fig. 1C). pTRE-SN-VP16 was therefore used to establish a stable Neuro-2a clonal cell line for Dox-inducible expression of SIMN-VP16 and ARNT2 (see "Experimental Procedures" for details). One clone (clone 37) with this characteristic was obtained. As shown in Fig. 2A, this clone expressed readily detectable levels of the SimN-VP16-IRES-Arnt2 transcript of the predicted size of 4.2 kb upon Dox treatment. The SIMN-VP16 and ARNT2 proteins were also detected (Fig. 2B). No SimN-VP16-IRES-Arnt2 transcripts or SIMN-VP16 and ARNT2 proteins were detectable with the wild-type CME, but not the mutant CME. Transfections, luciferase measurement, and normalization were the same as described for B. Arbitrary luciferase values were used to indicate CME activation.

Fig. 1. A, schematic representation of the plasmids used in this study: pTRE2hyg, empty vector without a cDNA insert used as a control; pTRE-SF, pTRE2hyg harboring full-length Sim1 and Arnt2 joined by the IRES; pTRE-SN-Gal4, pTRE2hyg harboring the Sim1 N-terminal bHLH/PAS domain (SimN) fused to the Gal4 activation domain, IRES, and Arnt2; pTRE-SN-VP16, pTRE2hyg harboring SimN fused to the VP16 activation domain, IRES, and Arnt2; pTRE-Arnt2 and pTRE-Sim-VP16, pTRE2hyg harboring Arnt2 and SimN fused to VP16, respectively; pTet-On, for expression of the tetracycline-controlled transactivator rtTA; pTet-tTS, for expression of the tetracycline-controlled silencer tTS; pML/6C-WT (17), a luciferase reporter driven by the adenovirus major late promoter linked to four copies of CME (4×CME) for SIM1/ARNT2 binding; pML/6C-AM (17), a similar reporter linked to four copies of CME with point mutations (CMEm) as shown. TRE, tetracycline-responsive element for rtTA and tTS binding; PCMV, cytomegalovirus-derived enhancer/promoter for high level transcription; PminCMV, minimal cytomegalovirus-derived enhancer/promoter. B, comparison of the activities of SIM1 variants in the Tet-On system. Transient transfections of the Neuro-2a cells were carried out using the plasmids indicated. The fold induction was calculated as relative luciferase activities of the doxycycline-treated (Dox+) over the mock-treated (Dox−) samples. The relative luciferase activities were normalized to the /H9252-galactosidase activity from cotransfected pSV-/H9252gal (data not shown). Results represent the mean of three independent experiments, and the error bars represent S.D. values.

Fig. 2. The stable Neuro-2a cell line clone 37 displays tightly regulated SIMN-VP16 and ARNT2 expression by Dox. Shown are the results from the Northern (A) and Western (B) analyses of the expression of SimN-VP16-IRES-Arnt2 mRNA and the SIMN-VP16 and ARNT2 proteins, respectively. Clone 37 cell lines were treated for 20 h under mock-induced (Dox−) and Dox-induced (Dox+) conditions before harvesting for total RNA and protein lysate. The SimN-VP16-IRES-Arnt2 transcript was detected by a [32P]dCTP-labeled Sim1 probe, whereas the SIMN-VP16 and ARNT2 proteins were detected by anti-VP16 and anti-ARNT2 antibodies, respectively (see "Experimental Procedures"). No visible background expression was detected in the mock-treated samples. In C, the clone 37 cell line showed Dox-induced activation of PMl/6C-WT reporter luciferase activity in a time-dependent manner. Strong activation of the reporter was seen between 16 (160-fold) and 24 (520-fold) h of Dox treatment. Three parallel samples were harvested at each time point. Results represent the mean, and the error bars represent S.D. values.
found under the mock-induced conditions (Fig. 2, A and B). For the temporally regulated transcription activity of SIMN-VP16/ARNT2 in the clone 37 cells, we determined the time course of pML/6C-WT reporter activity upon Dox treatment. After 8, 16, and 24 h of Dox treatment, the achieved induction of the reporter was 6-, 160-, and 530-fold, respectively (Fig. 2 C). Therefore, SIMN-VP16/ARNT2 transcription activity can be induced to high levels between 16 and 24 h of Dox treatment in the clone 37 cells.

Genes Regulated by SIMN-VP16/ARNT2 in the Microarray Analysis—We prepared total RNA from mock- and Dox-treated clone 37 cells in parallel. Northern analysis was used to determine the expression levels of the SIMN-VP16-IRES-ARNT2 transcript prior to subjecting the RNA to microarray analysis. The Affymetrix mouse chip MG-U74v2 A (containing 12,000 probe sets) was used. Independent probe syntheses from the same batch of RNA and hybridizations were performed in triplicate. The data sets were normalized, analyzed, and presented as a scatter correlation plot in Fig. 3B.
Downstream Targets of SIM1 and ARNT2

Positive domains in the hypothalamus. Five genes, Jak2 genes have expression domains overlapping with the used ISH to examine whether some of these potential target genes of NPAS2/BMAL1 in the cortex (25). Together, these two examples lend support to the general applicability of such an experimental design.

There are, however, limitations to this approach. First, chromatin accessibility of certain target genes may be limited in a given cell line. Second, the cell lines used may not provide tissue-specific accessory factors needed for SIM1/ARNT2 function. Third, there may be fortuitous activation of genes that contain CME in their promoters, but that are neither coexpressed with nor regulated by Sim1/Arnt2 in vivo. Although the first issue cannot easily be overcome, we minimized the other concerns by surveying various neuronal cell lines, modifying SIM1 by fusing it to the VP16 activation domain, and performing ISH as a secondary screen for in vivo coexpression and regulation.

Comparison of Microarray and Northern Data—Our results and those of others (26) demonstrated that the fold differences measured by Northern analysis are generally higher than those measured by microarray. Such a discrepancy may be due to inherent differences in these two methods. Yet, this observation raises the possibility that target genes displaying low threshold differences are unavoidably excluded by applying stringent statistical criteria to the microarray data. On the other hand, by choosing a 1.7-fold increase as a cutoff value for up-regulated genes, we found that 12 of the 15 genes tested that gave signals on the Northern blots are up-regulated. For the three genes that did not give signals, they may be false predictions, or they are in fact up-regulated, but expressed at too low a level for detection by Northern hybridization using total RNA. Based on these data, we expect that of the 268 genes, a similar percentage of them can also be confirmed independently.

Hypothalamic Expression of the Potential Downstream Genes—Several up-regulated genes selected for ISH show expression patterns overlapping with the Sim1 expression pattern. Specifically, these selected genes are expressed in discrete

The presence of the Arnt2 gene on this chip serves as an internal control of Dox induction. A total of 268 genes displaying >1.7-fold (arbitrarily chosen) increased expression were considered significantly up-regulated by t test, with p ≤ 0.05. These 268 genes can be divided into several functional categories as summarized in Fig. 3C and described in the legend thereof. The identity of each gene and its fold induction are listed in Table I. Importantly, no significant gene expression changes were found in the parental cell lines treated with Dox versus the mock-treated clone 37 cell line, indicating that the up-regulated genes in the Dox-treated clone 37 sample are regulated by SIMN-VP16/ARNT2.

Of note, we found that many of the up-regulated signaling component genes have been implicated in energy homeostasis (Table I), e.g. the MC3R, Jak2, and TRβ2 genes. Their possible roles in the Sim1/Arnt2-operated pathway will be discussed.

Independent Confirmation of the Microarray Results by Northern Analysis—We selected a group of target genes predicted by microarray analysis to perform Northern hybridization for independent confirmation. 15 genes were selected based on their possible relevance to the neuroendocrine system or obesity pathway (see Fig. 4 legend for the gene names). Total RNA samples of the mock- and Dox-treated clone 37 cells were subjected to Northern analysis using specific DNA fragments of these genes. Of the 15 genes, 12 were confirmed to be up-regulated upon Dox treatment. Fig. 4 shows 9 of the 12 confirmed examples. The other three were not confirmed, as they did not give a detectable signal (data not shown). Of note, the fold changes observed by Northern analysis were generally higher than those observed by microarray analysis.

Expression of Potential Sim1/Arnt2 Downstream Targets in the Neuroendocrine Hypothalamus—As a secondary screen, we used ISH to examine whether some of these potential target genes have expression domains overlapping with the Sim1-positive domains in the hypothalamus. Five genes, Jak2 and IL-6Rα (of the signaling component category), TRβ2 and Tlx2 (of the transcription regulator category), and Chrne (of the neurotransmitter receptor category), were chosen for this survey. ISH was carried out on sectioned E18.5 mouse brains using digoxigenin-labeled antisense probes of each gene. Adjacent sections throughout each brain were hybridized to the Sim1 probe and the gene probe of interest for comparison. The Sim1-positive regions are diagrammed in Fig. 5A (based on the data in Fig. 5B). In addition to its expression in the PVN and SON (10), Sim1 expression was also found in the nucleus of the lateral olfactory tract (NLOT) in the amygdala and in the anteroventral nucleus (AVN) in the hypothalamus (Fig. 5B).

When Jak2 expression was compared with that of Sim1 in adjacent sections, we noticed that Jak2 was expressed at the ventral PVN (Fig. 5D), occupying a subdomain of the Sim1 domain (Fig. 5C). Expression of Jak2 was localized to the mid-to-posterior level of the PVN. On the other hand, TRβ2 gene expression was found in the dorsal region of the PVN Sim1 domain at the posterior PVN level (Fig. 5, compare G and H). Expression of the IL-6Rα, Tlx2, and Chrne genes was also found at the mid-to-posterior PVN, and each displayed a distinctive subdomain expression pattern within the PVN Sim1 domain (Fig. 5, compare K–N). With the exception of Tlx2 (Fig. 5Q), expression of the other four potential Sim1 target genes was also found in the SON (Fig. 5, E, F, I, J, O, P, and R). All of these genes were also commonly expressed in the AVN and NLOT (Fig. 5, C–R). Sense probes of these genes gave no specific signals in the regions of interest: the PVN, (Fig. 5, C', D', G', H', L', M', and N') and SON and NLOT (Supplemental Fig. 1). Thus, of the five genes surveyed, all displayed expression domains overlapping with those of Sim1.

Expression of the Jak2 and TRβ2 Genes Is Down-regulated in the Sim1 Mutant—If these identified potential targets are true downstream genes of Sim1/Arnt2, we reasoned that their expression in Sim1 mutants should be significantly reduced or lost. We chose to examine Jak2 and TRβ2 gene expression in the Sim1 mutant by ISH. These two genes were chosen because of their potential relevance for hypothalamus-mediated energy homeostasis. Importantly, Sim1/Arnt2 is essential for PVN cell terminal differentiation, but not for cell genesis or survival. Sim1-positive cells are found in the Arnt2 mutant located laterally to the normal PVN position (3). In addition, in a lacZ knock-in allele of Sim1, LacZ-positive mutant cells were present in the same ventral location as the Sim1-positive cells in the Arnt2 mutant. In contrast to its expression in the wild-type PVN and AVN, Jak2 expression in the Sim1 mutant was greatly decreased in the presumptive PVN and AVN area (Fig. 6, compare A and B). Similarly, TRβ2 gene expression was greatly reduced in the presumptive PVN and AVN area in the Sim1 mutant (Fig. 6, compare C and D). This set of data suggests that Sim1 (and by inference, Arnt2) acts upstream of the Jak2 and TRβ2 genes.

DISCUSSION

From a Neuronal Cell Line to the Neuroendocrine System—Here, we used the Tet-On system in Neuro-2a cells to over-express SIMN-VP16/ARNT2 and screened for potential downstream genes of Sim1/Arnt2 in the neuroendocrine hypothalamus. An edcsyne-inducible system was also used recently in the neuronal SHEP cell line to obtain downstream genes of NPAS2/BMAL1 in the cortex (25). Together, these two examples lend support to the general applicability of such an experimental design.

There are, however, limitations to this approach. First, chromatin accessibility of certain target genes may be limited in a given cell line. Second, the cell lines used may not provide tissue-specific accessory factors needed for SIM1/ARNT2 function. Third, there may be fortuitous activation of genes that contain CME in their promoters, but that are neither coexpressed with nor regulated by Sim1/Arnt2 in vivo. Although the first issue cannot easily be overcome, we minimized the other concerns by surveying various neuronal cell lines, modifying SIM1 by fusing it to the VP16 activation domain, and performing ISH as a secondary screen for in vivo coexpression and regulation.

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### Table 1

Up-regulated genes from microarray analysis

The genes shown here are those whose fold increase was >1.7-fold in the microarray analysis. The fold induction change was determined by comparison of mean average difference scores. Differentially expressed genes were identified by Student’s t test (p < 0.05). ETS, expressed sequence tag; AhR, aryl hydrocarbon receptor; MMTV, murine mammary tumor virus; FGF, fibroblast growth factor.

| GenBank/EIBI accession no. | Gene name and description | Change |
|---------------------------|---------------------------|--------|
| **Transcription regulators** |                           |        |
| U89489                    | LIM domain binding 2 (Ldb2) | 1.74   |
| AW125812                  | SRY box-containing gene 10 (Sox10) | 1.78   |
| Y03836                    | Reversion-induced LIM gene (Ril) | 1.81   |
| M85566                    | Zinc finger protein 36, CSH type-like 1 (Zfp36l1) | 1.81   |
| AF152935                  | Musculin (Msc) | 1.81   |
| AB025922                  | GLI-Krüppel family member GLI1 (Gli1) | 1.82   |
| U36576                    | Nuclear factor of activated T-cells, cytoplasmic 2 (Nfasc2) | 1.82   |
| AB81574                   | CCR4-NOT transcription complex, subunit 7 (Cnot7) | 1.84   |
| AV214622                  | POU domain, class 5, transcription factor 1 (Pou5f1) | 1.85   |
| D13684                    | Osteohlast-specific factor 2 (Ostf2) | 1.88   |
| AA864065                  | Core-binding factor-β (Cbfb) | 1.93   |
| D49658                    | LIM homeobox protein 8 (Lhx8) | 1.93   |
| AV369921                  | Early growth response 1 (Egr1) | 2.01   |
| U61110                    | Eyes absent homolog 2 (Drosophila) (Eya1) | 2.02   |
| M75953                    | T-cell leukemia homeobox 2 (Tlx2) | 2.11   |
| L38622                    | Transcription regulator, SIN3 yeast homolog B (Sin3b) | 2.12   |
| AJ228069                  | Transcription factor 3 (Tcf3) | 2.12   |
| AF007110                  | Transformed mouse 3T3 cell double minute 4 (Mdm4) | 2.14   |
| AV369922                  | Nuclear receptor subfamily 5, A1 (Nr5a1) | 2.15   |
| Z35294                    | Mature T-cell proliferation 1 (MTCP-1) | 2.18   |
| AFO89242                  | Nuclear antigen Sp100 | 2.19   |
| U90535                    | Forkhead box B1 (Foxh1) | 2.3    |
| Z36885                    | Member of ETS oncogene family (ELK4) | 2.36   |
| U36799                    | Retinoblastoma-like 2 (Rbl2) | 2.4    |
| A8956111                  | Nascent polypeptide-associated complex α-polypeptide (Napa) | 2.43   |
| L09600                    | Nuclear factor, erythroid-derived 2 (Nfε2) | 2.48   |
| A843911                   | Glucocorticoid-induced leucine zipper (Gilda) | 2.51   |
| AV377676                  | GATA-binding protein 2 (Gata2) | 2.56   |
| U19140                    | Factor in the germine α (Fgα) | 2.58   |
| X13945                    | Lung carcinoma myc-related oncogene 1 | 2.71   |
| AB006192                  | MAD homolog 3, Drosophila (Snaf3, Dr . . .) | 2.73   |
| AW2120428                 | General transcription factor III A (Glf3a) | 2.77   |
| U155458                   | Thyroid hormone receptor β2 (Tребета-2) | 2.9    |
| U46186                    | Zinc finger protein 93 (Zfp93) | 2.91   |
| L80874                    | NK6 transcription factor-related 2 (Nkx6-2) | 3.06   |
| AV317359                  | SWI/SNF-related subfamily d, member 2 (Smarcd2) | 3.08   |
| AB842926                  | SWI/SNF-related subfamily e, member 1 (Smarce1) | 3.54   |
| D89844                    | AhR receptor nuclear translocator (Arnt2) | 4.17   |
| **Signaling components**  |                           |        |
| AI289693                  | Angiopoietin-like 4 (Angptl4) | 1.70   |
| U02567                    | Tumor necrosis factor receptor superfamily, member 9 (Tnfrsf9) | 1.73   |
| X007971                   | Interleukin-5 | 1.83   |
| AW050346                  | Neuronal guanine nucleotide exchange factor (Ngef) | 1.84   |
| AF078112                  | Calcium signal-modulating ligand (Camlg) | 1.87   |
| M98902                    | Wingless-related MMTV integration site 7B (Wnt7b) | 1.88   |
| AB0109281                 | Neurogenin B receptor | 1.88   |
| X71789                    | Burkitt lymphoma receptor 1 (Blr1) | 1.94   |
| K01238                    | Interferon-α family gene (Ifna2) | 1.95   |
| X74993                    | Melanocortin-3 receptor | 2.00   |
| AJ223777                  | Striatin, calmodulin-binding protein (Strn) | 2.01   |
| AB009250                  | FGF-17 | 2.02   |
| X01973                    | Interferon-α family gene 4 | 2.05   |
| AA123848                  | Regulator of G-protein signaling 10 (Rgs10) | 2.05   |
| AV246464                  | Janus kinase 2 (Jak2) | 2.06   |
| AF093257                  | Homer, neuronal immediate-early gene 1 (Homer1) | 2.10   |
| U78525                    | Chemokine-like receptor 1 (Cmkr1) | 2.12   |
| X78687                    | Ryanodine receptor 2 (Ryr2) | 2.14   |
| A0113385                  | Decidual/trophoblast prolatin-related protein (Dtprp) | 2.15   |
| X90778                    | LIF receptor gene | 2.18   |
| M90388                    | Protein-tyrosine phosphatase, non-receptor type 8 (Ptpn8) | 2.26   |
| X09569                    | Fc receptor, IgG, low affinity III (Fgrc3) | 2.77   |
| U25219                    | Angiogenin-related protein gene (Angpr) | 2.29   |
| D78188                    | Myotrophin (Mtpn) | 2.30   |
| D84196                    | Tumor necrosis factor | 2.31   |
| L33412                    | Advanced glycosylation end product-specific receptor (Ager) | 2.37   |
| X09063                    | Delta-like 1 homolog (Drosophila) (Dll1) | 2.39   |
| AFO24637                  | TYRO protein-tyrosine kinase-binding protein (Tyrobp) | 2.42   |
| M18656                    | T-cell receptor β, joining region (Tcrb-J) | 2.52   |
| U17252                    | G-protein-coupled receptor, family C, group 1, member H (Gprch) | 2.54   |
| L08594                    | Epidermal growth factor receptor | 2.66   |
| X51975                    | Interleukin-6 receptor α (Il6ra) | 2.73   |
| X76066                    | Insulin-like growth factor-binding protein 4 (Igfibp4) | 2.73   |
| U29678                    | Chemokine (C-C) receptor 1 (Ccr1) | 2.79   |
### Downstream Targets of SIM1 and ARNT2

| Gene | Description | Fold |
|------|-------------|------|
| Z46845 | Glucagon (Gcg) | 2.83 |
| A042654 | Hepatocyte growth factor activator (Hgfac) | 2.96 |
| V0741 | Epidermal growth factor | 3.05 |
| L15435 | Tumor necrosis factor (ligand) superfamily, member 9 (Tnfsf9) | 3.10 |
| AA914345 | Interferon-inducible GTPase (Igi) | 3.18 |
| X03278 | T-cell receptor β joining region (Tcrb-J) | 3.21 |
| U22516 | Angiogenin (Ang) | 3.23 |
| AF052607 | Non-receptor tyrosine kinase gene (Tyk2) | 3.35 |
| AF019926 | Serine/threonine kinase 22B (Stk22b) | 3.83 |
| U5713 | Phospholipase Cβ1b (Plcb1) | 4.03 |
| AV296394 | Glial cell line-derived neurotrophic factor (Gdnf) | 4.33 |

#### Metabolic enzymes

- AV267263: Copper chaperone for superoxide dismutase (CcS)
- AF079565: Ubiquitin-specific protease UBP41 (Ubip41)
- AI593999: Zymogen granule membrane protein 16
- AJ009682: Deoxyribonuclease 1 (Dnase1)
- AW129909: N-Acetylated α-linked acidic dipeptidase 2 (Naatald2)
- AF017639: Carboxypeptidase X2
- AW046124: Cytochrome b5 (Cyba)
- Z84471: Gpd-2 gene
- X65259: Ubiquitin-activating enzyme E1, Chr X (Ubex1)
- U10410: Recombinant anti-neuraminidase single chain Ig V domain
- M60803: Glutamine synthetase (Glns)
- AB035174: Sialytransferase 7D (Sia7T)
- AV039241: Testis-specific gene 1 (Tsps1)
- X68164: Sialytransferase 8 (α-2, 8-sialyltransferase) (Sia8T)
- U13705: Cytochrome b5 (Cyba)
- U73819: Galactosamine-polypeptide N-acetylgalactosaminytransferase 4 (Galnt4)
- AV277568: Thioredoxin reductase 3 (Txnr3)
- U66573: Phospholipase A2, group V (Pla2g5)
- AA571586: NADH dehydrogenase (ubiquinone) 1 α-subcomplex, 1 (Ndula1)
- AB013849: Peptidylarginine deiminase type III (Padi3)
- Z72905: Nac1 gene for N-acetyltransferase
- AF027301: Guanylyl cyclase C
- X65501: Glutathione S-transferase 1 (Gst1a)
- M62953: Cytochrome c, testis
- AV003213: UDP glycosyltransferase 1 family, polypeptide A6 (Ugtla6)
- D32250: Aldose reductase (Akrb3)
- AB017482: Aldehyde oxidase 1 (Aox1)
- AB050982: Isoprenylcysteine carboxylmethyltransferase (Icmr)
- M15268: Aminolevulinic-acid synthase 2, erythroid (Ams2)
- AV212241: Ornithine decarboxylase antizyme (Oaz1)

#### Channels and transporters

- U86823: Aquaporin 4 (Aqp4)
- U700688: Potassium voltage-gated channel, subfamily Q, member 1 (Kcnn1)
- A1842212: Customer protein complex, subunit 2 (Cops2)
- AJ131398: Potassium voltage-gated channel, Isk-related family, member 1-like (Kcne1)
- J03298: Lactotransferrin (Lf)
- AJ01107: Cdc1 gene, chloride channel gene
- AI826288: Cholinergeric receptor, nicotinic, α-polypeptide (Chrrn)
- AB010100: Aquaporin 7 (Aqp7)
- X51986: α-Aminobutyric acid A receptor, subunit α6
- AF028071: Calcium-binding protein D9k (Calc3)
- AJ006306: Skeletal muscle calcium channel, γ-subunit (Cacng1)
- AB028804: Synaptotagmin 7 (Synt7)
- X77241: Solute carrier family 17, member 1 (Slc17a1)
- X65473: Cholinergeric receptor, muscarinic 4
- X73985: Calbindin 2 (Calb2)
- AB028806: Synaptotagmin IX
- U61085: Solute carrier family 12, member 3 (Slc12a3)
- X62933: Tachykinin receptor 2 (Tacr2)
- AA96758: Sorting nexin 5 (Snx5)
- AB047120: Glutamate receptor, ionotropic, NMDA1 (1) (Grin1)
- AF085696: Inward rectifier K+ channel 4.2 (Kir4.2)

#### Cell adhesion- and migration-related genes

- AV283483: Erine (or cysteine) proteinase inhibitor, clade A, member 5 (Serpin5)
- X78218: Semaphorin G
- U04751: Formin-binding protein 1 (Fhbp1)
- D38162: Procollagen, type XI, α1 (Col11a1)
- U12147: Laminin, α2 (Lama2)
- M17997: Epidermal growth factor-binding protein type A
- U42898: Laminin, β3 (Lamb3)
- AF057156: Small proline-rich protein 1A (Sprr1a)
- U60146a: A disintegrin and metalloprotease domain 28 (Adam28)
- AV366904: Fk506-binding protein 3 (Fkbp3)
- U52652: Procollagen, type XII, α1 (Col12a1)
- X06340: Cadherin 3 (Cdh3)
- AF045573: Leucine-rich repeat (in FLII)-thretoacting protein 1 (Lrfip1)
- AV206509: Integrin β3 (Itgb3)
| Miscellaneous          | Fold |
|------------------------|------|
| AF047699               |      |
| Y155092                |      |
| AV329184               |      |
| J03257                 |      |
| AV366872               |      |
| D57837                 |      |
| AV361541               |      |
| AV335882               |      |
| AV377480               |      |
| X70383                 |      |
| D89992                 |      |
| AW016309               |      |
| AW058578               |      |
| A1842472               |      |
| AI154281               |      |
| M63697                 |      |
| M11900                 |      |
| AV119058               |      |
| M15585                 |      |
| AV130885               |      |
| M33226                 |      |
| Z24722                 |      |
| X01697                 |      |
| L09241                 |      |
| AV243986               |      |
| A1839906               |      |
| AW060861               |      |
| X16423                 |      |
| U969779                |      |
| AV049898               |      |
| M17962                 |      |
| Y15376                 |      |
| M76599                 |      |
| A1849827               |      |
| Y18289                 |      |
| A1785422               |      |
| U38456                 |      |
| M16472                 |      |
| AW060861               |      |
| A1197161               |      |
| AV233170               |      |
| X14655                 |      |
| M27134                 |      |
| AW047616               |      |
| AJ018134               |      |
| M19911                 |      |
| M26385                 |      |
| M33036                 |      |
| AA492742               |      |
| U394356                |      |
| M16472                 |      |
| AW060861               |      |
| A1197161               |      |
| AV233170               |      |
| X14655                 |      |
| M27134                 |      |
| AW047616               |      |
| AJ018134               |      |
| M19911                 |      |
| M26385                 |      |
| M33036                 |      |
| AA492742               |      |
| U394356                |      |
| M16472                 |      |
| AW060861               |      |
| A1197161               |      |
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| AW060861               |      |
| A1197161               |      |
| AV233170               |      |
| X14655                 |      |
| M27134                 |      |
| AW047616               |      |
| AJ018134               |      |
| M19911                 |      |
| M26385                 |      |
| M33036                 |      |
| AA492742               |      |
| U394356                |      |
| M16472                 |      |
| AW060861               |      |
| A1197161               |      |
| AV233170               |      |
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| AW047616               |      |
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| M19911                 |      |
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| M33036                 |      |
| AA492742               |      |
| U394356                |      |
| M16472                 |      |
| AW060861               |      |
| A1197161               |      |
| AV233170               |      |
| X14655                 |      |
| M27134                 |      |
| AW047616               |      |
| AJ018134               |      |
| M19911                 |      |
| M26385                 |      |
| M33036                 |      |
| AA492742               |      |
| U394356                |      |
| M16472                 |      |
| AW060861               |      |
| A1197161               |      |
| AV233170               |      |
| X14655                 |      |
| M27134                 |      |
| AW047616               |      |
| AJ018134               |      |
| M19911                 |      |
subdomains of the PVN, which may reflect the diverse cell types residing in the PVN. Four of the five genes examined display both SON and PVN expression, consistent with the fact that some neurons in the SON and PVN have the same developmental origin (27, 28). To our surprise, all five genes are also commonly expressed in the AVN and NLOT, where Sim1 is also expressed. Although the physiological functions of these two nuclei are unknown, our data suggest that they are developmentally or functionally linked to the PVN and SON. In addition, the extensive correlation of expression between these genes and Sim1 strongly argues for their regulation by Sim1 in vivo.

Regulation of Hormone Genes by Sim1 and Arnt2—Although the oligonucleotides representing the OT, VP, and TRH genes are on the chip, they are not found in the up-regulated gene group. This is likely due to the fact that they are specialized markers for mature secretory neurons, and their chromatin organizations are not accessible in Neuro-2a cells. We are, however, surprised that Brn2 was not up-regulated by both microarray and Northern analyses (data not shown). Shh was also not in the up-regulated pool (data not shown), even though Sim1 overexpression activates ectopic Shh expression in transgenic mouse embryos (29). The lack of Shh and Brn2 in the up-regulated pool suggests that, although our approach is fruitful, it does not uncover all in vivo targets.

Among the five hormone genes (VP, OT, CRH, TRH, and somatostatin) whose expression is missing in Sim1 and Arnt2 mutants, none has been shown to be a direct target of Sim1/Arnt2 (2–5, 19). It is likely that Sim1 and Arnt2 govern a second tier of transcription factors, which in turn act independently or in combination to direct each hormone gene expression. For example, Brn2 acts downstream of Sim1/Arnt2, and its gene product can bind and activate the CRH promoter (22). On the other hand, the TRH promoter can be activated by TRβ2 in a thyroid hormone-independent manner in cultured cells (30, 31). Our finding that the TRβ2 gene is a target of Sim1/Arnt2 suggests that Sim1/Arnt2 utilizes the TRβ2 gene to activate the TRH gene during development. In addition, our screen uncovered 38 potential Sim1/Arnt2 targets in the transcription factor category. They may be employed by Sim1/Arnt2 to orchestrate the expression of lineage-specific hormone genes such as OT, VP, and somatostatin.

Genes Involved in the Energy Homeostasis Pathway—Sim1 heterozygotes develop early onset obesity. It has been proposed that PVN hypodevelopment of embryonic origin instead of PVN malfunction of adult origin is the contributing cause (6). We were therefore surprised to find that several genes directly implicated in the negative regulation of body weight (but not in PVN development) turn up in the up-regulated pool, including the MC3R, Jak2, TRβ2, and IL-6Ra genes. The MC3R gene mediates the melanocortin-stimulating hormone signaling pathway, and MC3R mutant mice develop obesity (32, 33). Jak2 is thought to be a positive participant in leptin receptor signaling (34, 35). Both melanocortin-stimulating hormone and leptin are well known negative regulators of food intake (36). Although their actions have been investigated in the arcuate

| Sequence  | Description  | fold |
|-----------|--------------|------|
| AI661057  | Unknown      | 2.01 |
| AI449297  | Unknown      | 2.04 |
| C797238   | Unknown      | 2.04 |
| AV252456  | Unknown      | 2.07 |
| AV003017  | Unknown      | 2.08 |
| AA517864  | Unknown      | 2.09 |
| AA516738  | Unknown      | 2.09 |
| AR41211   | Unknown      | 2.10 |
| C78513    | Unknown      | 2.13 |
| AI841295  | Unknown      | 2.16 |
| AW122431  | Unknown      | 2.17 |
| AA408991  | Unknown      | 2.20 |
| AW125538  | Unknown      | 2.21 |
| AV053298  | Unknown      | 2.25 |
| W31672    | Unknown      | 2.26 |
| AV314618  | Unknown      | 2.36 |
| AV256386  | Unknown      | 2.40 |
| R75193:MDB1137 | Unknown | 2.46 |
| C80266    | Unknown      | 2.49 |
| AI035317  | Unknown      | 2.53 |
| C78704    | Unknown      | 2.57 |
| AA408385  | Unknown      | 2.59 |
| AA795946  | Unknown      | 2.59 |
| C78923    | Unknown      | 2.61 |
| AI462038  | Unknown      | 2.64 |
| C81463    | Unknown      | 2.64 |
| AW060190  | Unknown      | 2.65 |
| C79709    | Unknown      | 2.68 |
| T25656    | Unknown      | 2.69 |
| AI854358  | Unknown      | 2.72 |
| AV217226  | Unknown      | 2.86 |
| AA915720  | Unknown      | 2.91 |
| C76510    | Unknown      | 2.95 |
| AV105090  | Unknown      | 2.99 |
| C80498    | Unknown      | 3.08 |
| AI846824  | Unknown      | 3.08 |
| AW229127  | Unknown      | 3.09 |
| AV087536  | Unknown      | 3.22 |
| AI852734  | Unknown      | 3.79 |
| AI852933  | Unknown      | 3.79 |
| AI826942  | Unknown      | 3.92 |
| C79030    | Unknown      | 4.06 |
| AW045507  | Unknown      | 5.80 |
Genes in this class may facilitate the structural formation of the PVN (and in part SON) because they fail to congregate centrally and migrate laterally, and PVN mutants stay at a lateral position (with respect to the normal PVN) because they fail to congregate centrally and migrate laterally. This may be because genes also have important implications. As mentioned above, the cell adhesion and migration category acts to confer or regulate the neuronal connectivity of neurons therein. Together, these two categories of genes may help to build the structural and functional PVN and SON.

In IL-6R mutant mice also develop obesity (37). Expression of the IL-6R gene increases and the obesity phenotype observed.

**Potential Target Genes of Other Functional Categories—** There are many potential target genes of Sim1/Arnt2 in other functional categories. Genes such as Chrne, Grin1, and Homer1 are involved in neurotransmitter receptor (38–40). Their potential downstream status supports the possibility that Sim1/Arnt2 acts to confer or regulate the neuronal connectivity of PVN and SON cells. The cell adhesion and migration category of genes also has important implications. As mentioned above, the presumptive PVN and SON cells in the Sim1 and Arnt2 mutants stay at a lateral position (with respect to the normal PVN) because they fail to congregate centrally and migrate laterally to form the anatomical PVN and SON, respectively.

Genes in this class may facilitate the structural formation of the PVN and SON as well as axonal projections of the neurons therein. Together, these two categories of genes may help to build the structural and functional PVN and SON.

It is puzzling that metabolic enzymes such as glucose-6-phosphate dehydrogenase and NADH dehydrogenase are in the up-regulated pool. The function and tissue-restricted expression of Arnt2 and Sim1 do not support their general role in regulating metabolic enzyme levels. We suggest that these genes are targets of another bHLH/PAF protein pair(s) with
JAK2, which are also found in similar locations in the human mouse sequence (ACGTG) near their loci. Within 10 kb upstream of the Sim1 presumptive posterior PVN level in the E18.5 mutant. The microarray data analysis. For the sim1 mutant, there is one CME site located within the microarray data analysis. For the sim1 mutant, there is one CME site located within the microarray data analysis. For the sim1 mutant, there is one CME site located within the microarray data analysis. For the sim1 mutant, there is one CME site located within the microarray data analysis. For the sim1 mutant, there is one CME site located within the microarray data analysis. For the sim1 mutant, there is one CME site located within the microarray data analysis. For the sim1 mutant, there is one CME site located within the microarray data analysis. For the sim1 mutant, there is one CME site located within the microarray data analysis. 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