also pBiPs were enriched for CRE consensus sequences, and were preferential targets for regulation by the transcription factors in the cAMP-dependent pathway (Figures 3B and 8 and Supplementary Figure S13). We reported previously that transcription of pancRNAs from pBiPs epigenetically activates partner tissue-specific genes (7,8,11). Consistent with previous reports that pancRNAs contribute to the establishment of cell identity via epigenetic regulation of tissue-specific gene expression, the expression levels of pancRNAs derived from pBiPs tended to be positively correlated with the expression levels of their partner mRNAs during differentiation of PC12 cells (Figure 3C). Furthermore, epigenetic activation via pancRNAs was confirmed here by the manipulation of the *pancNusap1* levels in PC12 cells (Figures 5–7). Thus, the present results provide the new insight that transcriptional regulation by means of pancRNAs provides adaptive advantages for CRE-mediated gene regulation. Considering that the number of pBiPs is almost 6-fold greater than the number of aBiPs, the impacts of pBiPs as important effectors of cAMP signals might be greater than those of aBiPs for coordinated activation of a fraction of cAMP-responsive genes.

The silencing of M-phase progression contributes to the establishment of an irreversibly differentiated state: implications for neuronal differentiation

We showed that regulation of cell-cycle-associated genes plays crucial roles in the process of irreversible differentiation of PC12 cells (Figures 5–7). The critical importance of maintaining the cell-cycle-arrested state in neurons is supported by studies on somatic cell nuclear transfer. When nuclei of adult cortical neurons are used as donors, the majority of reconstructed mouse oocytes are arrested at the first mitotic cleavage with abnormal chromosome condensation (40), whereas reconstructed oocytes cloned from nuclei of other types of differentiated somatic cells, such as cumulus cells (41) and tail tip fibroblasts (42), show much greater developmental potential. Because the cell cycle of neurons is arrested at G0 phase, many studies have focused on the relationship between neuronal differentiation and inhibition of G1/S-phase progression (43,44). For example, neuronal differentiation of mouse neural stem cells is enhanced by double knockout of G1/S-phase-progression-associated genes *Cdk2* and *Cdk4* (45). Similarly, Ndiff PC12 cells have been widely used as an *in vitro* model for investigating the relationship between G1/S-phase inhibition and neurite outgrowth. In fact, like the Cdk2 protein level, the level of Pcna and phosphorylated retinoblastoma protein, also known as G1/S-phase-progression-associated proteins, are downregulated in Ndiff PC12 cells (46–48), suggesting that G1/S-phase inhibition also occurs in reversibly differentiated Ndiff PC12 cells.

In addition, several lines of evidence indicate that inhibition of M-phase-associated genes is involved in the functional differentiation of neurons (49,50). For example, reconstructed mouse oocytes cloned from nuclei of adult cortical neurons undergo abnormal cell cycle arrest at the first mitotic cleavage (40). In mouse neural progenitors, KD of an M-phase-progression-associated gene, doublecortin-like kinase, causes neuronal differentiation (51). Therefore, coordinated regulation of G1/S-phase-progression-associated genes and M-phase-progression-associated genes seems to be important for neuronal differentiation. In this context, it is interesting to note that the expression of cell-cycle-associated genes, especially those related to G2/M-phase progression, remained high in Ndiff PC12 cells (Figure 2) and epigenetic silencing of M-phase-associated *Nusap1* could convert reversibly differentiated cells into irreversibly differentiated cells (Figure 5). Considering that inhibition of G1/S-phase progression is observed in reversibly differentiated Ndiff PC12 cells (Supplementary Figure S14), NGF and cAMP signalings appear to be strongly involved in G1/S- and G2/M-inhibition, respectively, for completing the differentiation of neurons. Our results suggested that irreversible/reversible PC12 differentiation could be a good model for further addressing the lncRNA-mediated mechanisms that link cell cycle regulation to irreversible differentiation.

The pancRNA repertoires are differentially utilized from the zygotic to the terminally differentiated stages

In this study, the RNA-seq method was utilized to test whether pancRNA-mediated gene activation mechanisms function in the terminal differentiation of the cells on a genome-wide scale, and the results confirmed this hypothesis (Figures 3, 5 and 6). Indeed, pancRNAs have been detected from more than a thousand promoter regions at a very early stage of life called zygotic gene activation (7). Previous reports have also shown that thousands of pancRNAs are transcribed in ES cells and various tissues (11,52). Thus, a non-negligible number of pancRNAs seem to be expressed in various contexts. Since pancRNAs and mRNAs exhibit coordinated expression changes not only in totipotent/pluripotent cells but also in terminally differentiated cells (Figure 3C), pancRNAs may be commonly utilized for gene activation from the zygotic to the terminally differentiated stage. Although we focused on a study of functional pancRNAs from the polyA+ and >200 bp ncRNA fraction in this study, other fractions of ncRNAs (e.g. polyA-, <200 bp) derived from the promoter of protein-coding genes may also function in transcriptional activation of their partner genes (37,53). The general usage of the pancRNA system over the course of life sheds light on the importance of gene-activation-associated ncRNAs as counterparts of gene-silencing ncRNAs, such as microRNA, which work at the post-transcriptional level. Studies of possible coordinated regulation with lncRNAs derived from enhancer regions, called eRNAs (32,54–59) would be the next step in elucidating the sequence-specific gene activation mechanisms in which ncRNA structurally recognizes genomic DNA.

Transcriptional silencing of *Nusap1* in response to extracellular differentiation stimuli is an important step of cAMP signaling toward terminal differentiation of PC12 cells

Nusap1 protein has a well-conserved microtubule-binding domain in its COOH terminus and a DNA-binding SAP domain in its NH2 terminus, and promotes the stabilization and crosslinking of microtubules near chromosomes

in metaphase/anaphase (60,61). *Nusap1* is highly expressed in proliferative tissues, and essential for M-phase progression. In cultured cell lines, KD of *Nusap1* causes severe mitotic defects with defective anaphase and cytokinesis (60), and knockout of *Nusap1* induces early embryonic lethality in mice (62). Like other cell cycle regulators, the protein level of Nusap1 is under the regulation of a multisubunit E3 ubiquitin ligase, anaphase promoting complex/cyclosome, in proliferating cells (63). However, the mechanisms of transcriptional regulation of *Nusap1* in terminally differentiated cells are still unknown. In this study, we showed that reduced transcription of *Nusap1* via downregulation of *pancNusap1* in response to cAMP stimulation functions in the terminal differentiation of PC12 cells (Figures 5–8). These findings indicated that transcriptional silencing of *Nusap1* is a key mediator of cAMP signaling for terminal differentiation of PC12 cells. The epigenetic silencing by the concomitant reduction of *pancNusap1* by cAMP may further support the complete shut-down of neuronal cell division.

Possible molecular mechanism underlying *pancNusap1*-mediated epigenetic modification

We and others have reported that pancRNA promotes the assembly of an open chromatin conformation that includes histone acetylation, H3K4 methylation, H3K9/K27 demethylation and active DNA demethylation (7,8,12,64). Differently from these previous results, the present data indicated that the activation and reduction of *pancNusap1* expression increased and decreased the histone acetylation levels of the *Nusap1* promoter, respectively, but not the levels of histone methylation or DNA methylation (Figures 4–6, and Supplementary Figure S1). There are many reports indicating that functional lncRNAs recruit epigenetic modification complexes, such as histone methyltransferase myeloid/lymphoid or mixed-lineage leukemia (MLL) complex and the BER components at specific genomic locations (1,56,65–67). However there are no reports that prove the interaction of lncRNAs with histone acetyltransferase complex. Recently, nuclease-null Cas9-based transcriptional activation technology has revealed that artificial transcription initiation at an intergenic locus elevates H3K27ac levels (68). Furthermore, single living cell analysis has shown that recruitment of RNAPII occurs within a few minutes after histone acetylation at the glucocorticoid receptor gene locus (69). Given these reports, one possible scenario is that transcription initiation of *pancNusap1* primary causes histone acetylation at the *Nusap1* promoter and thus enhances specific gene expression there.

Interestingly, we and others have reported that only antisense pancRNAs are associated with chromatin, and pancRNA-mediated transcriptional activation occurs in a strand-specific manner (8,64). Consistent with these reports, the transcriptional direction of *pancNusap1* is important for *pancNusap1*-mediated histone acetylation (Figure 6). In this context, it is worthwhile to note that *pancNusap1* has a G-rich sequence (Supplementary Figure S15). Because guanine (G)-rich RNA that hybridizes with single-stranded cytosine (C)-rich complementary DNA forms a thermodynamically stable DNA:RNA hybrid structure, known as an R-loop structure (70,71), the sequence-dependent stability of R-loops may explain the increase of histone acetylation by *pancNusap1*. Further studies will be needed to unravel the molecular mechanisms that determine the sequential epigenetic changes induced by pancRNA-mediated histone acetylation.

In conclusion, pancRNA-mediated histone acetylation contributes to the establishment of PC12 cells' stimulation-induced transcription status during the irreversible differentiation process.

DATA AVAILABILITY

RNA-seq data have been deposited in the DDBJ Sequence Read Archive (DRA) under accession number DRA004148.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Identification of genes associated with the astrocyte-specific gene *Gfap* during astrocyte differentiation

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Kenji Ito[1], Tsukasa Sanosaka[2], Katsuhide Igarashi[3], Maky Ideta-Otsuka[3], Akira Aizawa[1], Yuichi Uosaki[1], Azumi Noguchi[1], Hirokazu Arakawa[1], Kinichi Nakashima[2] & Takumi Takizawa[1]

Chromosomes and genes are non-randomly arranged within the mammalian cell nucleus, and gene clustering is of great significance in transcriptional regulation. However, the relevance of gene clustering and their expression during the differentiation of neural precursor cells (NPCs) into astrocytes remains unclear. We performed a genome-wide enhanced circular chromosomal conformation capture (e4C) to screen for genes associated with the astrocyte-specific gene glial fibrillary acidic protein (*Gfap*) during astrocyte differentiation. We identified 18 genes that were specifically associated with *Gfap* and expressed in NPC-derived astrocytes. Our results provide additional evidence for the functional significance of gene clustering in transcriptional regulation during NPC differentiation.

An increasing amount of evidence supports the importance of spatial organization of the genome in the nuclei of higher eukaryotes[1]. Chromosomes and genes are non-randomly arranged and occupy preferential positions within the nucleus[2]. Moreover, these arrangements are associated with gene regulation because the sub-nuclear positions of genes change along with alterations in their transcriptional states[3–6]. For instance, in naïve $CD4^+$T helper cells, there is an inter-chromosomal association between the regulatory region of the TH2 cytokine locus and the interferon γ (*Ifng*) promoter region, where both are repressed[7]. On the other hand, in erythroid cells, Klf1-regulated genes including globins preferentially associate at a limited number of transcriptional factories containing high levels of Klf1 once activated[8]. Other observations based on chromosome conformation capture (3C) and its derivative techniques (4C, 5C, ChIA-PET) have shown that gene associations play roles in transcriptional regulation[9–12]. These techniques are essential for revealing three-dimensional information regarding the spatial proximity of DNA within the cell nucleus[13,14].

Neural precursor cells (NPCs) in the central nervous system can self-renew and differentiate into neurons mid-gestation, and then into astrocytes and oligodendrocytes only after late-gestation[15]. Differentiation of NPCs is temporally and spatially regulated by several factors including cytokines and epigenetic modifications[16,17]. NPCs from mouse telencephalon at late gestation (e.g., embryonic day [E] 14.5) are competent to differentiate into astrocytes upon stimulation with leukemia inhibitory factor (LIF)[18,19]. LIF activates the transcription factor STAT3, which then binds to the promoter of an astrocyte specific gene, glial fibrillary acidic protein *(Gfap)*, to induce its expression[19,20]. This is of great relevance in astrocytogenesis, since mice lacking a common receptor for LIF, gp130, are largely devoid of *Gfap*-positive astrocytes. These astrocytes show a lower ability to support the survival of neurons[21]. In addition, DNA demethylation and chromatin remodeling in STAT3 binding motifs on the *Gfap* promoter are essential for *Gfap* expression[22].

Gfap gene loci have been shown to undergo a shift toward a more internal location upon transcriptional activation[6]. Furthermore, genomic regions adjacent to nuclear lamina are replaced as gene expression programs

[1]Department of Pediatrics, Graduate School of Medicine, Gunma University, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan. [2]Stem Cell Biology and Medicine, Department of Stem cell Biology and Medicine, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan. [3]Life Science Tokyo Advanced Research Center (L-StaR), Pharmacy and Pharmaceutical Science, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-5801, Japan. Correspondence and requests for materials should be addressed to T.T. (email: takizawt@gunma-u.ac.jp)

change during astrocyte differentiation from NPCs[23]. This indicates robust conversion of genome localization during astrocytogenesis; however, little is known about the relevance of gene clustering in NPC differentiation.

In this study, we screened for genes that associate with *Gfap* during the astrocyte differentiation of NPCs by using enhanced circular chromosome conformation capture with minor modifications (modified e4C). We looked for a correlation between gene clustering and transcriptional activities by comparing data from modified e4C and expression arrays. We identified 18 genes associated with *Gfap* that are also expressed specifically in LIF-induced astrocytes. DNA florescence *in situ* hybridization (FISH) confirmed the clustering of some genes and *Gfap*. These findings support the possibility that the association of co-expressing genes is involved in astrocyte differentiation.

Results

Genome-wide screening of genes specifically associated with *Gfap* and expressed in NPC-derived astrocytes. As a first step toward identifying genes clustered with and regulated similarly to *Gfap* during astrocyte differentiation, we decided to perform a modified e4C assay with a few modifications[8]. NPCs derived from E14.5 mouse brains can differentiate into astrocytes after being cultured *in vitro* for more than 4 days in the presence of the astrocyte-inducing cytokine LIF[19]. We isolated neuroepithelial cells from the telencephalon of E14.5 mice and cultured them for 5 consecutive days (designated as NPCs). After one passage, the NPCs were further cultured for 4 days with LIF to differentiate them into astrocytes (designated as LIF+ cells) (Fig. 1A). As reported previously, under these conditions, ~20% of NPCs differentiate into astrocytes as judged by immunofluorescence labeling of the astrocyte marker *GFAP* (Fig. 1B)[6,19]. The NPCs grown in extended culture without LIF (LIF− cells) were also tested as a control (Fig. 1A,B).

As "bait" for the e4C assay, we used a genomic region containing a STAT3 cognitive sequence on the *Gfap* promoter, the *Gfap* STAT3-binding site (GSBS). The GSBS is located ~1.5 kb upstream of the transcription start site and is a prerequisite for *Gfap* transcription during astrocytogenesis from NPCs[19]. We first tried *Bgl*II digestion of the flanking regions of GSBS. However, the digestion efficiency in this region as assessed with quantitative PCR was much lower in cells that did not express *Gfap* than those that did express *Gfap* (20.8% vs. 61.3%). We assumed the insufficient digestion was due to highly compacted chromatin around the GSBS in those cells[22]. To improve accessibility of restriction enzymes to the chromatin, we added an extra step of hydrochloric acid treatment to the original e4C protocol (Fig. 1C). Indeed, this achieved comparable digestion efficiency at the GSBS region in different types of cells (Fig. 1D) and helped to identify a large number of e4C peaks in both *cis* and *trans* in two biological replicates. As expected, many peaks were found around the bait locus, and the ratio between the e4C peak number and chromosome size was the highest for chromosome 11, which encodes *Gfap* in all the types of cells tested (Fig. 1E,F).

Trans-chromosomal interactions were also found in all types of cells tested (Fig. 2A). A number of positive probes were not replicated between two biological repeats (Figure S1A); this might be partly due to a resolution difference between the 3C assay and microarray probes. The former sometimes has cytological but not necessarily molecular resolution[24], while the arrays represent molecular resolution and contain a probe or two for most target fragments between *Bgl*II and *Nla*III with expected lengths within 200 bp (Figure S1B). Therefore, we only focused on replicated positive probes. The analysis of distances of positive probes from the nearest genes revealed that probes 10–30 kbp away from the transcriptional start or end sites were enriched with e4C compared to all probes on the array (Figure S2). Indeed, as many as ~1,000 genes were found within 50 kbp from the positive probes that we defined as being associated with *Gfap* in each cell type (Fig. 2B and Supplemental Table S2), likely because of the relatively lower resolution of the 3C or its derivative assay[24]. Among them, 587 genes were associated with *Gfap* exclusively in LIF+ cells (Fig. 2B). We also found considerable overlap (564 genes) between NPCs and LIF− cells but less overlap between NPCs and LIF+ cells (430 genes) or LIF+ and LIF− cells (295 genes). These results show distinct associations between *Gfap* and other genes in LIF+ cells versus NPCs and LIF− cells.

To identify genes that are simultaneously associated with *Gfap* gene loci and expressed specifically in LIF+ cells, gene expression profiles in each cell type were examined by Affymetrix GeneChip analysis. To normalize gene expression levels from different samples, we adopted the Percellome method, which provides "per cell" read-outs in copy numbers of mRNA by adding a set of external spike mRNAs in proportion to the DNA content as a substitute for the measurement of cell numbers in the sample[25]. We found 2,083 genes in LIF+ cells and 2,404 in LIF− cells, with more than a two-fold increase in expression in NPCs. There were 1,132 overlapping genes between LIF+ and LIF− cells and 951 genes exclusively expressed in LIF+ cells (Fig. 2C and Supplemental Table S3). By referring to genes associated in LIF+ cells in the e4C assay, we found 18 genes that were associated with *Gfap* and expressed at particularly high levels in LIF+ cells (Table 1 and Fig. 2D).

Validation of GeneChip array and modified e4C results. Among the putative 18 genes, we selected *Ogn*, *Osmr*, *Ecrg4*, *A2m*, and *Gab1* for further analysis because they have been implicated as playing important roles in the central nervous system[26–30]. To affirm the results of the GeneChip analysis, we tested the expressions of the five genes and *Gfap* by RT-qPCR. As expected, mRNA expression levels of the six genes were all significantly increased 72 and 96 h after LIF stimulation (Fig. 3).

The long-range interactions identified by 4C technology need to be verified by completely independent methods such as FISH because 4C technology shows average chromosome conformations from millions of nuclei[31,32]. Therefore, we next performed DNA FISH for each of the five putative associating genes (Fig. 4A and Figure S3). In addition to these five genes, we also tested genes that were negative in modified e4C and located on different chromosomes, namely *Ahnak* on chromosome 19 and *Nme8* on chromosome 13. The GeneChip analysis showed that *Ahnak* is exclusively expressed in LIF+ cells, while *Nme8* is expressed at miniscule levels in NPCs, LIF+, and LIF− cells. Three genes (*Ogn*, *Osmr*, and *Ecrg4*) out of the five putative genes show a significant increase in the number of cells with associated alleles in the LIF+ culture (Fig. 4B). Although not significant, similar tendencies

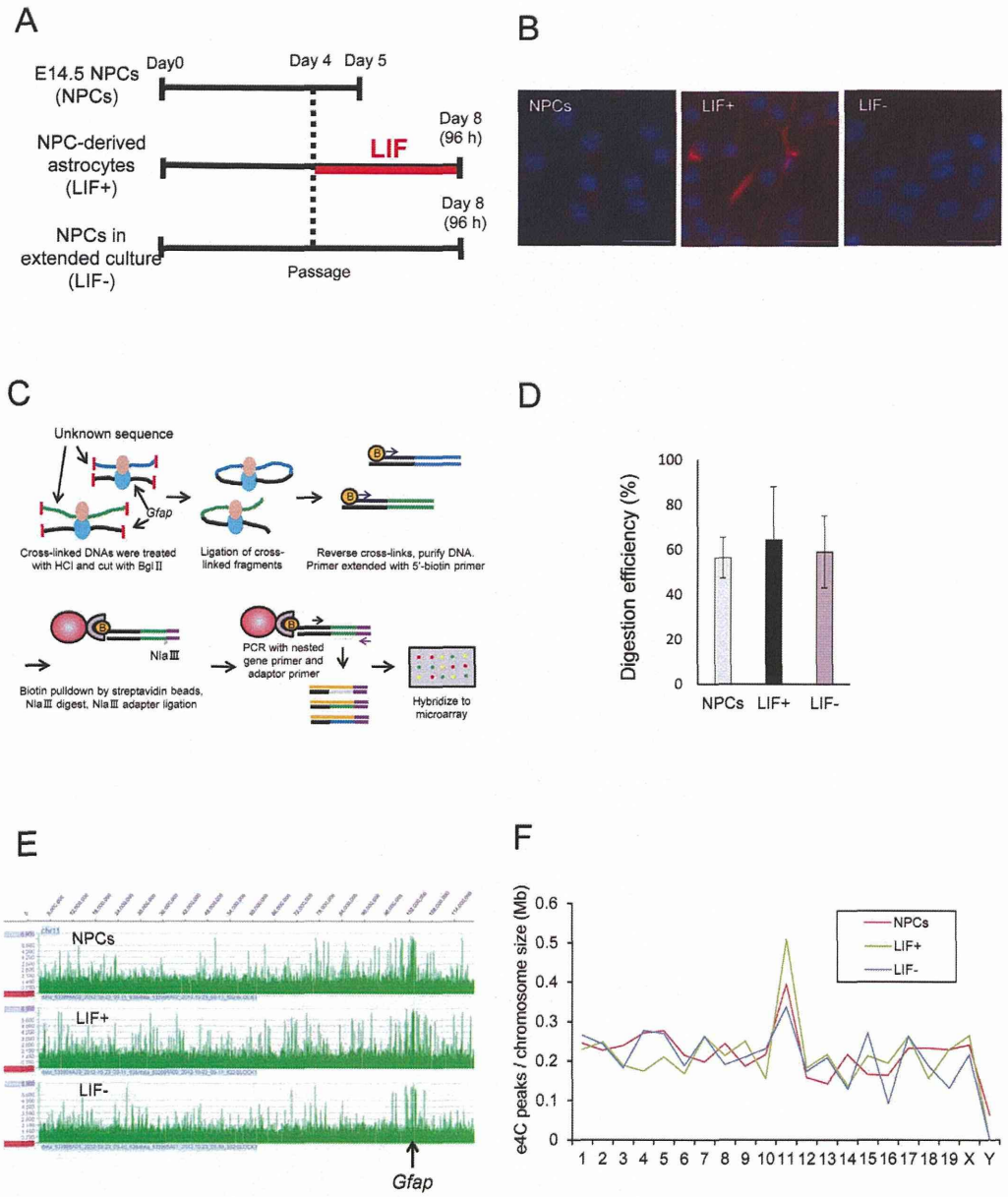

Figure 1. Genome-wide interactions of the *Gfap* loci in NPCs, LIF+, and LIF− cells. (**A**) Schematic experimental protocol. NPCs isolated from E14.5 mouse telencephalon were cultured and replated on day 4. On day 5, cells were used for experiments as NPCs. NPC-derived astrocytes and NPCs in extended culture were collected after an additional 4 days of culture with or without LIF. On day 8, the cells were used for experiments as LIF+ or LIF− cells. (**B**) NPCs, LIF+, and LIF− cells were stained with an anti-*GFAP* antibody (red, *GFAP*; blue, DAPI). Scale bar = 20 μm. (**C**) Schematic representation of the modified e4C method. Chromatin was fixed in paraformaldehyde and treated with 0.1 N hydrochloric acid, then digested with *Bgl*II and ligated. The resulting hybrid molecules were used as a template for the primer extension reaction using the bait-specific primer. This was followed by adaptor ligation, nested PCR, labelling with fluorescent dye, and hybridization to a custom microarray. (**D**) The digestion efficiency of the DNA samples used for modified e4C. Relative amounts of PCR products on a *Bgl*II recognition site located near the *Gfap* STAT3 binding site (GSBS) are shown. (**E**) Association profiles were determined as the signal ratio of e4C samples to reference genomic DNA. Log_2 (e4C DNA/genomic DNA) = 2 was set as a cut-off value. (**F**) Number of e4C peaks on each chromosome. Chromosome sizes were obtained from the Mouse Genome Browser Gateway (NCBI37/mm9).

were observed for *A2m* and *Gab1*. The number of cells with close proximity to *Gfap* did not change significantly for *Ahnak* and *Nme8* in different types of cells. Overall, there were no significant differences in the distribution of distances between *Gfap* and those gene loci among the three types of cells (Figure S4). We did notice a

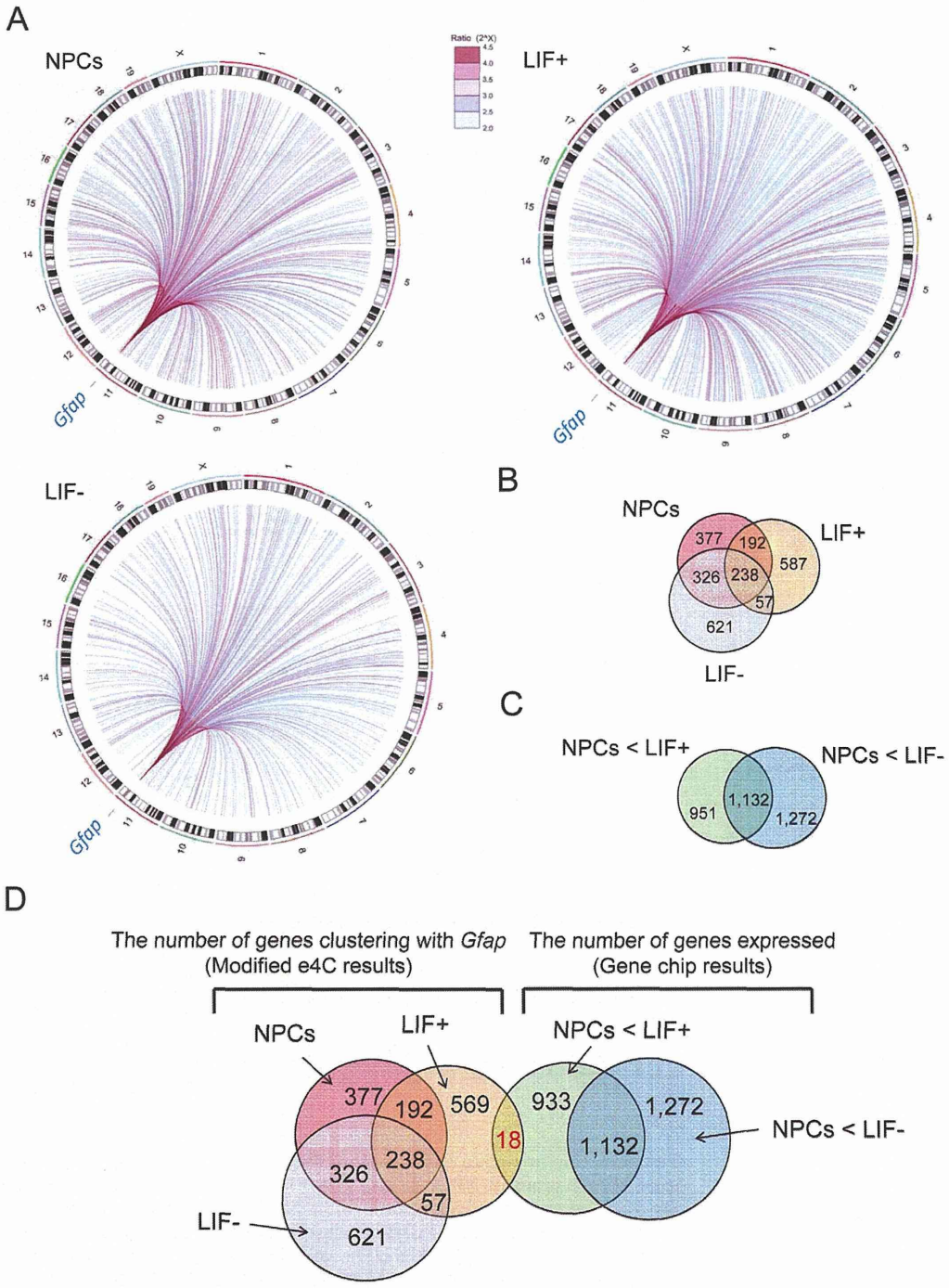

Figure 2. Identification of putative genes specifically associated with *Gfap* and expressed in LIF+ cells. (**A**) Circos plots showing interactions between the *Gfap* locus and interacting partners, with each line representing an interaction. Chromosomes are plotted along the circle. These plots were generated using the R package. The results of NPCs, LIF+, and LIF− cells are shown separately. Each line color represents the ratios of intensities of peaks compared to genomic controls. (**B**) Venn diagram of the *Gfap*-associating *Bgl*II fragments from e4C results in NPCs, LIF+, and LIF− cells. (**C**) Venn diagram of the genes expressed more highly in either LIF+ or LIF− cells than NPCs. (**D**) Venn diagram of 587 genes specifically associated with *Gfap* in LIF+ cells and 946 genes specifically expressed in LIF+ cells.

consistent difference between NPCs and LIF− in the association of each pair of probes (Fig. 4B). This was not explained by the nuclear diameter because it was not significantly different between NPCs, LIF+, and LIF− cells

Accession	Gene symbol	NPCs	LIF+	LIF−	LIF+/NPCs	LIF−/NPCs	STAT3 binding site	Distance to probe region detected by e4C
NM_011019	*Osmr*	0.20	14.43	0.27	72.14	1.33	○	10–30 kb from TES
NM_008760	*Ogn*	0.10	3.46	0.04	34.58	0.42	○	10–30 kb from TSS
NM_011313	*S100a6*	1.20	29.50	1.27	24.58	1.06	○	10–30 kb from TES
NM_172471	*Itih5*	0.60	11.04	1.81	18.40	3.02	○	>30 kb from TES
NM_175459	*Glis3*	0.40	2.97	0.38	7.43	0.94	○	>30 kb from TSS
NM_008046	*Fst*	0.20	1.36	0.23	6.82	1.16	○	>30 kb from TES
NM_133832	*Rdh10*	1.20	4.97	1.76	4.14	1.47	○	5–10 kb from TSS
NM_024283	*Ecrg4*	0.50	1.76	0.08	3.52	0.16	○	>30 kb from TSS
NM_011883	*Rnf13*	2.70	9.12	5.38	3.38	1.99	○	10–30 kb from TES
NM_175628	*A2m*	11.10	37.32	4.31	3.36	0.39	○	5–10 kb from TES
BC019423	*Rsph9*	0.80	1.99	0.61	2.48	0.76	×	10–30 kb from TES
BC110634	*Galntl1*	3.00	7.42	3.95	2.47	1.32	○	>30 kb from TES
NM_009011	*Rad23b*	6.00	14.72	11.27	2.45	1.88	×	>30 kb from TES
NM_009371	*Tgfbr2*	1.00	2.33	0.29	2.33	0.29	○	>30 kb from TSS
NM_173876	*Clcn3*	3.30	7.38	6.31	2.24	1.91	○	<2 kb from TES
NM_175836	*Spnb2*	4.60	10.06	6.84	2.19	1.49	○	>30 kb from TSS
NM_008654	*Ppp1r15a*	1.00	2.14	1.34	2.14	1.34	×	>30 kb from TES
BC094659	*Gab1*	11.70	23.66	11.72	2.02	1.00	×	2–5 kb from TES

Table 1. List of the 18 putative genes specifically associated with *Gfap* and expressed in LIF+ cells. TSS; transcription start site, TES; transcription end site.

(10.4 ± 1.5 μm in NPCs, 10.1 ± 1.1 μm in LIF+, and 10.2 ± 1.2 μm in LIF−). LIF− is an extended culture of NPCs, which readily differentiate into astrocytes with changes in epigenetic programs dedicated to astrocytes[19,33]. This might explain the constant difference between NPCs and LIF−. Consequently, these cell-based analysis results agree with the e4C findings.

Detailed gene association analysis. We previously reported that *Gfap* is expressed in a random monoallelic manner in LIF+ cells and cortical astrocytes[6]. Hence, we investigated whether the alleles that associate are preferentially expressed with a simultaneous RNA/DNA-FISH assay. We used a probe targeting mature *Gfap* transcripts and probes targeting *Gfap* and *Ogn* gene loci. A significantly larger number of active *Gfap* alleles associated with *Ogn* loci than inactive ones (Fig. 5A,B). The results suggest that *Gfap* transcriptional activity is at least partially correlated with gene association.

According to the position weight matrix of STAT3 derived from the previous ChIP-seq data[34], there are STAT3 binding motifs within 5 kb of the transcription start sites of 14 of the 18 genes screened with the e4C (Table 1). This indicates that these genes are activated by the common transcription factor, STAT3. We therefore aimed to determine whether the genes with STAT3 binding motifs share the same locale, i.e. a transcription factory. We performed a multicolor DNA FISH assay using probes targeting *Gfap*, *Ogn*, and *Osmr*. We did not observe these three genes clustered in our system (Fig. 5C,D), indicating that these gene associations did not simultaneously occur.

In addition, a detailed analysis of the e4C results did not indicate direct interaction among the promoters of those genes; positive probes for those 18 genes were not mapped on the promoter regions (Table 1).

Discussion

In this study, we identified 18 genes that are specifically expressed and associated with the astrocyte-specific gene *Gfap* in NPC-derived astrocytes (LIF+ cells) by comparing gene association data obtained by applying a modified e4C genome-wide screening method to genome-wide gene expression profiles obtained from a microarray analysis. Importantly, these results were reproducible with two additional independent and distinct methods: DNA FISH and RT-qPCR. We also showed that the association of these genes correlated with *Gfap* transcriptional activity (Fig. 5A,B).

Long-range chromatin interactions including gene clustering are increasingly being recognized as an important mechanism to regulate gene expression. In many instances, these multi-gene complexes are hypothesized to be organized within "transcription factories" containing RNA polymerase II (RNAPII) and other components involved in transcription[5,8,35]. Furthermore, most genes in these transcription factories are transcribed cooperatively, and some of these genes can influence each other[36]. Although we need to investigate further to reveal the functional significance of clustering, the 18 genes identified in this study may have roles in regulating *Gfap* expression and astrocyte differentiation. In fact, one of the identified genes, *Osmr,* encodes an oncostatin M (OSM) receptor known to be involved in STAT3 activation and subsequent *Gfap* expression during astrocyte differentiation[37]. The OSMR is essential for OSM, a member of the interleukin (IL)-6 family of cytokines, to activate downstream JAK-STAT signaling pathways by forming a heterodimer with the common signal transducer gp130. Interestingly, *Osmr* itself is transcriptionally activated by STAT3[38,39]. This suggests that STAT3 may be involved in gene associations between *Gfap* and *Osmr,* and that the association has roles in initiating enhanceosomes for

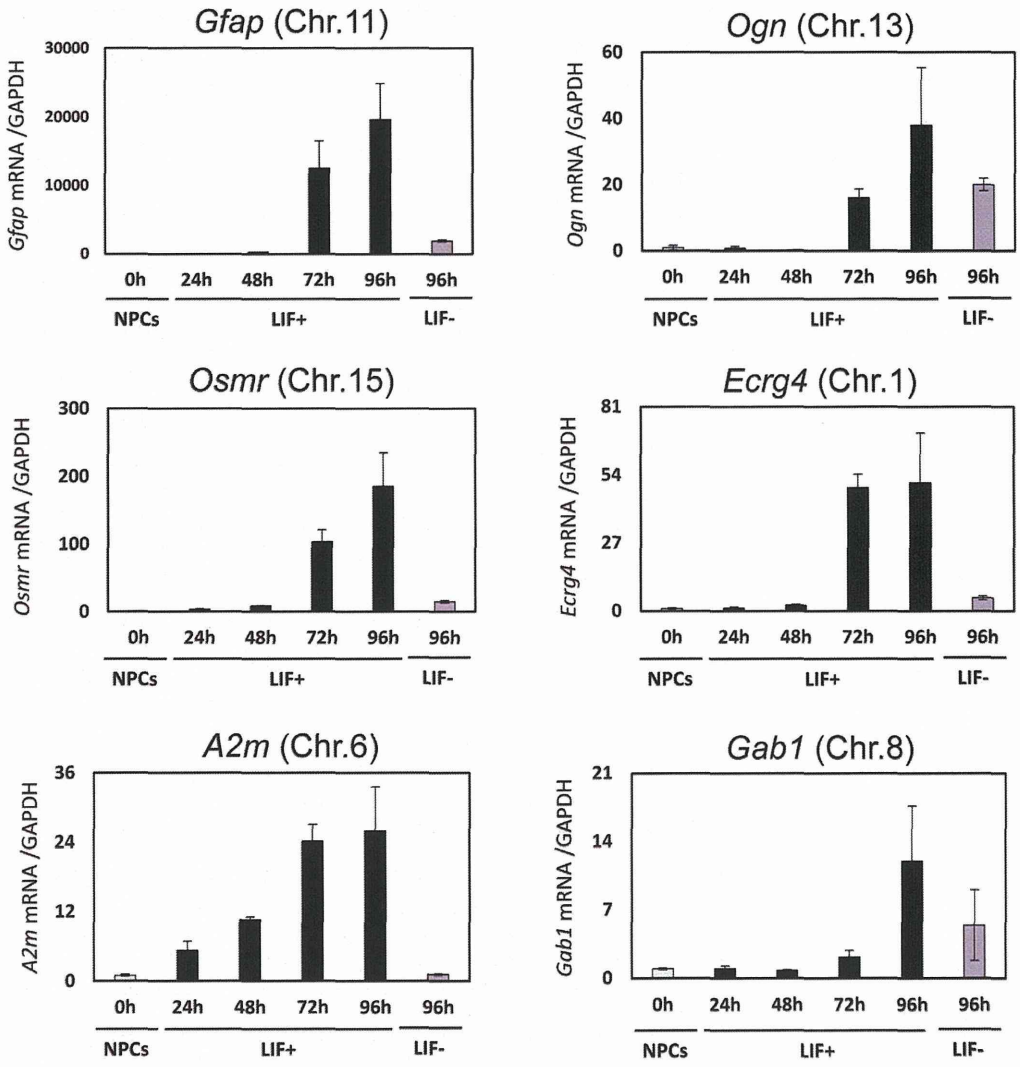

Figure 3. Validation in expression of *Gfap* and five putative genes identified by Genechip and modified e4C. Quantitative RT-PCR was performed on *Gfap* and five genes selected from modified e4C. The expression level of each gene was determined in NPCs (NPCs 0 h), cells stimulated with LIF for different periods of time (LIF+ 24 h, 48 h, 72 h, 96 h), and without LIF (LIF− 96 h). The results were normalized to *Gapdh* expression. Each graph represents the mean (±SEM) relative amount (compared with NPCs) in at least three experiments.

Gfap expression and astrocyte differentiation. Another identified gene, *Ecrg4,* participates in NPC cell cycle arrest through a mechanism involving the proteasome-dependent degradation of cyclins D1 and D3[27,40]. Gene associations between *Gfap* and *Ecrg4* may be relevant for the appropriate timing of transcription initiation and astrocyte differentiation. On the otherhand, e4C-positive probes were not found around the STAT3 binding sites of the 14 genes that possessed them. This indicates that close proximity of those genes, but not direct association of STAT3 binding sites, may play a role in enhancing transcription. It might be interesting to perform higher molecular resolution tests such as sequential chromatin immunoprecipitation to study gene interactions.

Changes in gene clustering reflect a wide range of genome functions such as replication, imprinting, and transcription[41–43]. Although we revealed transcription-related gene associations of *Gfap*, transcription is not the only factor governing associations between *Gfap* and other genes. *Gfap* is monoallelically expressed in primary astrocytes and asymmetrically replicated, as are a number of other monoallelically expressed genes[6]. Furthermore, the reduction of transcription-repressive histone modifications (e.g., methylation of H3 at lysine 9 [H3K9me2, 3]) and the expansion of transcription-permissive histone modifications (e.g., methylation of H3 at lysine 4 [H3K4me3] and acetylation of H3 at lysine 9 [H3K9Ac] at GSBS) are important for *Gfap* expression[44–46]. These previous findings support the idea that changes in the pairing of associated genes may depend not only on transcriptional activities but also on replication timing and epigenetic modifications. In fact, during differentiation of embryonic stem cells (ESCs) to NPCs, switching of chromosomal domains between early and late replication in the S-phase results in changes in gene pairings[43]. Furthermore, proteins that bind to H3K27me3 and cause DNA