The actin family member Arp6 and the histone variant H2A.Z are required for spatial positioning of chromatin in chicken cell nuclei

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Summary

The spatial organization of chromatin in the nucleus contributes to genome function and is altered during the differentiation of normal and tumorigenic cells. Although nuclear actin-related proteins (Arps) have roles in the local alteration of chromatin structure, it is unclear whether they are involved in the spatial positioning of chromatin. In the interphase nucleus of vertebrate cells, gene-dense and gene-poor chromosome territories (CTs) are located in the center and periphery, respectively. We analyzed chicken DT40 cells in which Arp6 had been knocked out conditionally, and showed that the radial distribution of CTs was impaired in these knockout cells. Arp6 is an essential component of the SRCAP chromatin remodeling complex, which deposits the histone variant H2A.Z into chromatin. The redistribution of CTs was also observed in H2A.Z-deficient cells for gene-rich microchromosomes, but to lesser extent for gene-poor macrochromosomes. These results indicate that Arp6 and H2A.Z contribute to the radial distribution of CTs through different mechanisms. Microarray analysis suggested that the localization of chromatin to the nuclear periphery per se is insufficient for the repression of most genes.

Key words: Actin-related protein, Histone variant, Nuclear organization, Chromosome territory, Gene expression

Introduction

Various chromatin remodeling and histone modification complexes play key roles with respect to the organization of chromatin. Members of the actin family, which consists of actin and actin-related proteins (Arps), are essential components of some of these complexes (Chen and Shen, 2007; Dion et al., 2010; Meagher et al., 2009; Ohfuchi et al., 2006; Oma and Harata, 2011). Arp6 is predominantly localized in the nucleus and identified as an essential component of the SWR1/SRCAP chromatin remodeling complex (Mizuguchi et al., 2004; Wu et al., 2005; Yoshida et al., 2010), which catalyzes the replacement of nucleosomal histone H2A with the variant H2A.Z both in yeasts and vertebrates (Mizuguchi et al., 2004; Ruhl et al., 2006; Wong et al., 2007).

The spatial arrangement of chromatin in the nucleus adds a further level of organization to the physical structure of the genome. In vertebrates, functional regions of the genome are located in spatially compartmentalized regions of the nucleus, and interphase chromosomes form chromosome territories (CTs) that occupy discrete domains (Cremer et al., 2006; Misteli, 2007). CTs in the interphase nucleus usually have a radial distribution, in which each CT is part of a polarized arrangement from the center to the periphery of the nucleus (Boyle et al., 2001; Croft et al., 1999; Sadoni et al., 1999). The position of CTs within this polarized arrangement is correlated with gene density (Bridger and Bickmore, 1998; Cremer and Cremer, 2001; Gilbert et al., 2005). In chicken cells, the territories of gene-rich microchromosomes and gene-poor macrochromosomes are positioned in the center and periphery of the nucleus, respectively. The physiological significance of the radial distribution of CTs is supported by evidence that their arrangement is specific to cell and tissue types, and is altered during development (Meaburn and Misteli, 2007; Meshorer and Misteli, 2006), and in multiple types of cancer cells (Cremer et al., 2003; Meaburn and Misteli, 2008; Wiech et al., 2005).

Despite many recent studies of chromatin organization, information about the spatial arrangement of chromatin in the nucleus remains limited. Although Arps are suggested to be required for higher levels of chromatin organization (Chuang et al., 2006; Hu et al., 2008; Lee et al., 2007; Yoshida et al.,

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2010), it is still unclear about their function in the spatial positioning of CTs in the nucleus.

Results and Discussion

Impairment of the radial distribution of chromosome territories in Arp6-deficient cells

To investigate the cellular function of Arp6, we constructed and analyzed conditional knockout (KO) cells for Arp6 using the chicken DT40 B cell line (supplementary material Fig. S1A). In the KO cells, the expression of the *ARP6* gene was controlled by a tetracycline (tet)-repressible promoter. Northern and western blot analyses showed that the expression of Arp6 in *ARP6*-KO cells was reduced upon exposure to tet, and Arp6 protein became undetectable by 96 hr after the addition of tet (supplementary material Fig. S1B,C). Consistently, the *ARP6*-KO cells treated with tet proliferated as well as the cells not treated with tet until 96 hr (supplementary material Fig. S1D). FACS analysis revealed that the distribution of Arp6-deficient cells at each stage of the cell cycle was not significantly changed in comparison with wild-type cells, and that the number of dead cells did not significantly increase until 144 hr after the addition of tet (supplementary material Fig. S2).

Chicken chromosomes are classified on the basis of their size into macrochromosomes and microchromosomes, which are composed of gene-poor and gene-rich chromatin, respectively (Habermann et al., 2001; Tanabe et al., 2002a; Tanabe et al., 2002b). We used the three-dimensional fluorescence in situ hybridization (3D-FISH) to analyze the spatial distribution of macro- and microchromosome territories. These two types of chromosomes were visualized using two pools of probes, one designed to detect macrochromosomes (chromosomes 1–8 and Z) and the other to detect 21 pairs of microchromosomes. The pools of probes were hybridized with three-dimensionally preserved nuclei. As reported previously (Habermann et al., 2001; Tanabe et al., 2002a; Tanabe et al., 2002b), the macrochromosomes (red) were found predominantly at the periphery of the nucleus, and the microchromosomes (green) formed a continuous cluster that was located in the center of the nucleus in wild-type and control (*ARP6*-KO without tet) cells (Fig. 1, left panels). In contrast, in Arp6-deficient cells, the macro- and microchromosome CTs were dispersed discontinuously and their central and peripheral distributions had disappeared (Fig. 1, right panels). Although less than 10% of the wild-type and control cells had the impaired radial distribution, the percentages increased to 58% and 89% in Arp6-deficient cells (Fig. 1). This result indicates that Arp6 is required for the radial distribution of CTs.

The radial distributions of macro- and microchromosomes in the nuclei were analyzed quantitatively and statistically (Tanabe et al., 2002a; Tanabe et al., 2002b) (Fig. 2). On each graph, the horizontal axis shows the relative radii of the nuclear shells, and the positions 0% and 100% correspond to the center and periphery of the nucleus, respectively. The vertical axes represent the normalized relative DNA content of painted CTs in a given area. In the Arp6-deficient cells (Fig. 2), the macrochromosomes shifted inwards whereas the microchromosomes shifted outwards as compared to wild-type cells (Fig. 2). The Mann–Whitney *U*-test showed that these differences were statistically significant ($P<0.01$).

We previously found that the budding yeast Arp6 is required for the spatial arrangement of chromatin in the nucleus (Yoshida et al., 2010). Therefore, it is suggested that the contribution of Arp6 to the higher order arrangement of chromatin in the nucleus is evolutionarily conserved.

H2A.Z is also required for the radial distribution of CTs

To explain why Arp6-deficient cells impair the radial distribution of CTs, we focused on the histone variant H2A.Z, because we recently showed that Arp6 has an essential function in the SRCAP complex, which is required for the deposition of H2A.Z (Matsuda et al., 2010). Vertebrate H2A.Z has two isoforms (H2A.Z-1 and H2A.Z-2) that are encoded by two individual genes. In Arp6-deficient cells, the deposition of both H2A.Z

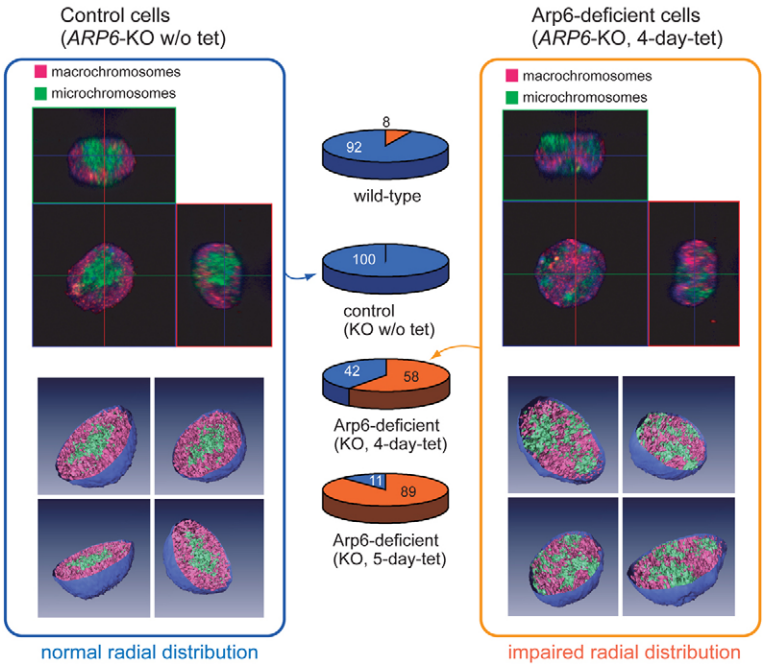

Fig. 1. Impairment of the radial distribution of chromosomes in Arp6-deficient cells. (Upper panels) 3D-FISH was performed on structurally preserved cell nuclei using probes for macrochromosomes (red) and for microchromosomes (green). Representative nuclei of control (*ARP6*-KO without tet) cells and Arp6-deficient (*ARP6*-KO with 4-day-tet) cells are shown. Front views and reconstituted side views of each nucleus are shown. (Lower panels) Three-dimensional reconstructions of the painted chromosomes were generated using the Amira 3.1 software. (Pie graphs) Nuclei with continuous clusters of microchromosome territories and with discontinuously dispersed microchromosome territories were counted as having normal radial distribution and impaired radial distribution, respectively. Nuclei in wild-type cells ($n=36$), *ARP6*-KO cells without tet treatment ($n=31$), and *ARP6*-KO cells with 4 days ($n=32$) or 6 days ($n=28$) of tet treatment were counted.

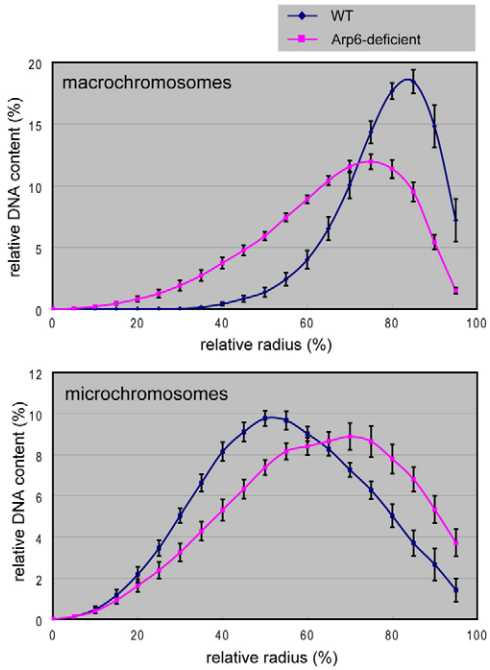

Fig. 2. Comparisons of the radial distributions of macro- and microchromosomes. The relative nuclear distributions of macrochromosomes (upper panel) and microchromosomes (lower panel) were compared in wild-type (n=34) and Arp6-deficient (n=36) cells. The error bars represent the standard error.

isoforms into chromatin was decreased to approximately 30% of that in wild-type cells. Although the isoforms have redundant functions, only H2A.Z-2 can prevent cells from entering apoptosis and can regulate the *BCL6* gene (Matsuda et al., 2010). We previously established a tet-repressible KO strain for *H2A.Z-1* (Matsuda et al., 2010). In the current study, the *H2A.Z-2* gene in this conditional *H2A.Z-1* KO strain was disrupted. In the resulting KO strain, only H2A.Z-1 was expressed in the absence of tet, and both H2A.Z-1 and H2A.Z-2 proteins became undetectable at 72 hr after the addition of tet (supplementary material Fig. S3A). H2A.Z-2-deficient cells (*H2A.Z*-KO cells without tet) proliferated at almost the same rate as wild-type cells throughout the course of the experiment. In contrast, in H2A.Z-deficient cells (*H2A.Z*-KO cells with tet for 72 hr), the number of living cells started to decrease at 96 hr (supplementary material Fig. S3B). This result is the first demonstration that both isoforms of H2A.Z have redundant functions in vegetative cell growth and the existence of one or the other is required for the cell survival.

3D-FISH analysis revealed that, in both H2A.Z-2- and H2A.Z-deficient cells, the radial distributions of macrochromosome territories (Fig. 3B, upper panel) and of microchromosome territories (Fig. 3B, lower panel) were impaired compared with those in wild-type cells. Given that a similar effect is seen in H2A.Z-1-deficient cells, although to a lesser extent (supplementary material Fig. S4), the results indicate that the decreased levels of the H2A.Z isoforms contribute to the impaired radial distribution of CTs. However, the effect of Arp6 deficiency on the radial distribution of macrochromosome territories was more severe than that of H2A.Z deficiency (Fig. 3B, upper panel), which suggests that Arp6 and H2A.Z contribute to the radial positioning of CTs through different mechanisms. Consistently, in budding yeast, Arp6

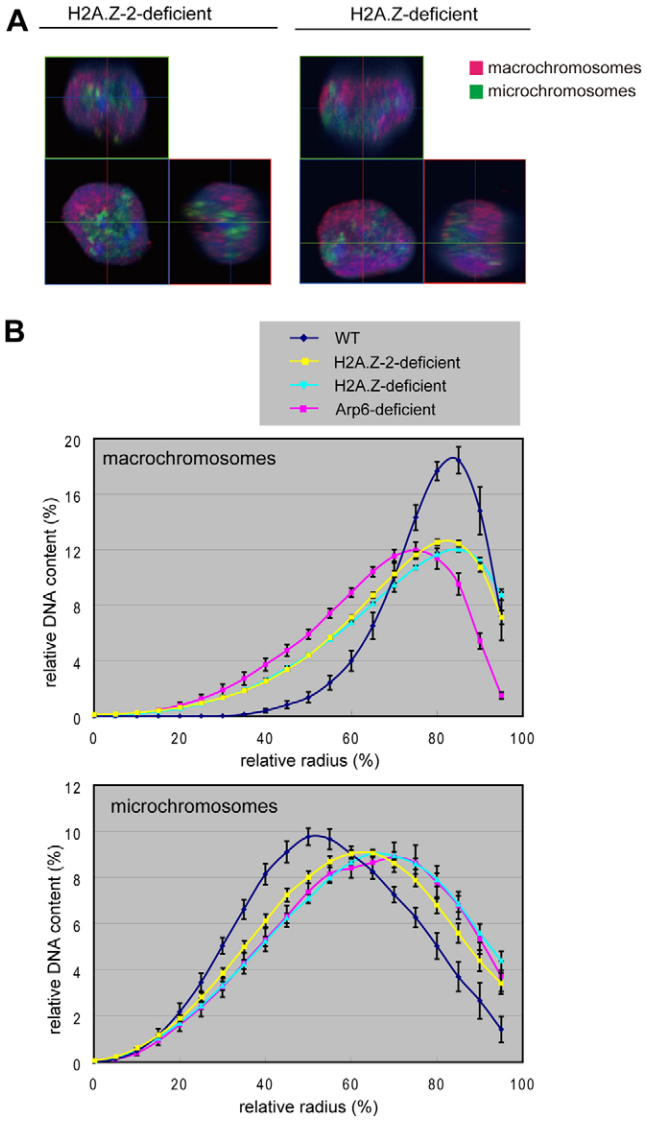

Fig. 3. Impairment of the radial distribution of CTs in H2A.Z-reduced and -deficient cells. (**A**) 3D-FISH (performed as in Fig. 1). (**B**) Comparisons of the radial distribution of macrochromosomes (upper panel) and microchromosomes (lower panel) in Arp6-deficient, H2A.Z-2-deficient and H2A.Z-deficient cells are compared with that in WT cells.

has also been shown to possess H2A.Z-dependent and -independent functions with respect to higher-order chromatin organization and transcriptional regulation (Yoshida et al., 2010). Given that the amount of the two H2A.Z isoforms is nearly identical in wild-type cells and deletion of either isoform induces only a slight upregulation of the other (Matsuda et al., 2010), it appears that H2A.Z-2, which is more highly conserved evolutionarily, makes a greater contribution to the radial distribution of CTs than H2A.Z-1. Importantly, the organization of CTs was also impaired in H2A.Z-1- and H2A.Z-2-deficient cells, even though the growth rate of these cells is similar to that of wild-type cells (supplementary material Fig. S3B) (Matsuda et al., 2010). This indicates that the defect in H2A.Z deposition, but not the subsequent decline in growth, affects the radial distribution of CTs. Interestingly, in budding yeast, the deposition of H2A.Z is required for the

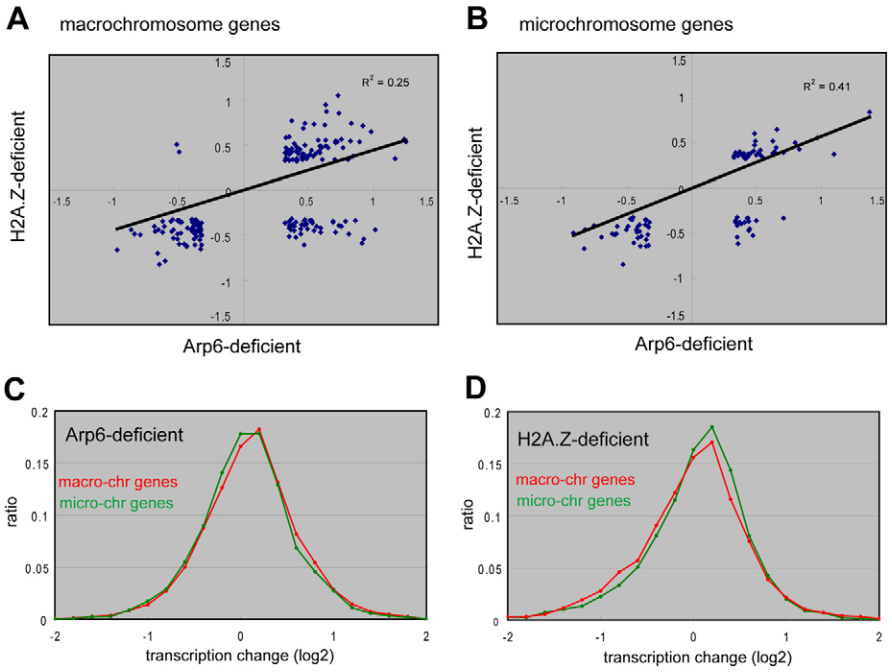

Fig. 4. Transcriptional misregulation in Arp6- and H2A.Z-deficient cells. (**A**,**B**) Log ratios of the change in transcription level of genes misregulated in Arp6- and H2A.Z-deficient cells (>1.25-fold). Genes on macrochromosomes and on microchromosomes are plotted in A and B, respectively. (**C**,**D**) The distribution of transcriptional changes for genes on macrochromosomes (red) and on microchromosomes (green) were compared in Arp6-deficient and H2A.Z-deficient cells, respectively.

localization of genome regions in nuclear periphery (Brickner et al., 2007; Kalocsay et al., 2009).

Transcriptional misregulation in Arp6- and H2A.Z-deficient cells

Recent observations indicate that the radial distribution of CTs affects the expression of a number of genes, but does not necessarily correlate with gene expression (Harewood et al., 2010; Küpper et al., 2007; Meaburn and Misteli, 2008). Similar results were obtained in 'tethering' experiments (Kumaran and Spector, 2008; Reddy et al., 2008). To examine the impacts of Arp6 and H2A.Z deficiencies on the transcription of genes, we performed a genome-wide microarray analysis. The raw data and experimental details have been deposited in the GEO database under accession number GSE14220 and 35430. We plotted the degree of change in the transcription level for each gene whose transcription was misregulated in both Arp6- and H2A.Z-deficient cells (>1.25-fold; Fig. 4). For both genes in macrochromosomes (Fig. 4A) and those in microchromosomes (Fig. 4B), the degree of misregulation showed a weak correlation between Arp6-deficient (horizontal axis) and H2A.Z-deficient (vertical axis) cells, and approximately 80% of the genes were plotted in either the upper right square (upregulated in both cell types) or the lower left square (downregulated in both cell types). This result indicates that Arp6 mostly plays a role in gene regulation through regulating the deposition of H2A.Z.

Then we compared the changes in transcription between macro- and microchromosome genes in Arp6- and H2A.Z-deficient cells. For both the Arp6- (Fig. 4C) and H2A.Z-deficient cells (Fig. 4D), the macro- and microchromosome genes were separated into two groups, their log ratios for the change in transcription were plotted, and the distributions compared. Obvious differences in the pattern of transcriptional changes between macro- and microchromosome genes were not detected in either type of deficient cell. This finding indicated that the impairment of the radial positioning of CTs did not cause global activation and repression due to the inward relocation of macrochromosome territories and outward relocation of microchromosome territories, respectively. Our findings seem to agree with previous observations that the localization of chromatin to the nuclear periphery per se is insufficient for the repression of most genes.

Possible contribution of Arp6 and H2A.Z to development and tumorigenesis through nuclear organization

Previous studies have shown that the intranuclear repositioning of chromosomal loci is linked to the differentiation of normal and tumorigenic cells (Cremer et al., 2003; Meaburn et al., 2009; Wiblin et al., 2005; Wiech et al., 2005). It has been observed that Arp6 and H2A.Z show dynamic behavior during development (Kato et al., 2001; Matsuda et al., 2010; Nashun et al., 2010). Interestingly, during early preimplantation development in mice, H2A.Z disappears temporarily and the absence of H2A.Z is required for normal development (Nashun et al., 2010). Therefore, it is an attractive hypothesis that Arp6 and H2A.Z are involved in regulation of the genome during development through their contribution to the spatial positioning of chromatin. Furthermore, human H2A.Z is being discussed as a target for cancer diagnosis and therapy (Rangasamy, 2010; Svotelis et al., 2010). It is clear that further analyses of Arp6- and H2A.Z-deficient cells will provide new insights into the contribution of the radial distribution of CTs to cell differentiation and malignancies.

Materials and Methods

Arp6- and H2A.Z-deficient cells

Targeted disruption constructs for the *ARP6* gene were generated such that genetic fragments encoding exons 6–11 were replaced with a histidinol (hisD)-resistance or puromycin (puro)-resistance cassette under the control of the β-actin promoter. The full-length cDNA for chicken Arp6 (Ohfuchi et al., 2006) was cloned into the *Bam*HI site of pUHD10-3 (Fukagawa et al., 2001) to yield a tet-repressible expression plasmid, pUHD-Arp6. To produce H2A.Z-deficient cells, the disruption construct for the *H2A.Z-2* gene was transfected into the *H2A.Z-1*$^{-/-/H2A.Z\text{-}1\ transgene}$ cell line (Matsuda et al., 2010).

3D-FISH

A detailed protocol for 3D-FISH is described elsewhere (Solovei et al., 2002). The probes were originally obtained from flow-sorted chicken chromosomes (Griffin et al., 1999). Two pools of probes were prepared: pool 1, which detected 21 pairs of microchromosomes (total 42), was labeled with biotin; pool 2, which detected the macrochromosomes 1–8 and Z, was labeled with digoxigenin. The hybridization conditions, including post-hybridization washings and detection procedures, were essentially the same as described previously (Habermann et al., 2001). Nuclei were scanned with an axial distance of 200 nm using a three-channel laser scanning confocal microscope (LSM510 META, Carl Zeiss, Oberkochen, Germany). Three-dimensional reconstructions of image stacks for the CTs were generated by using AMIRA 3.1 TGS software. The nuclear periphery was reconstructed from thresholded images of the DNA counterstain.

3D-RRD

The relative radial distance (RRD) program is designed to quantify the average position of fluorescent marked objects with respect to the nuclear center and the nuclear border in many cell nuclei (Tanabe et al., 2002a; Tanabe et al., 2002b). For this purpose, the program subdivided the nuclear space into, for example, 20 concentric shells that covered the entire volume of the nucleus. For each point within the nucleus, two lines were drawn and their lengths determined; the first line was from the center of the nucleus to the given point and the second line was from the center to the border of the nucleus through the point. The ratio of the lengths of the two lines denoted the relative position of the point with respect to the nuclear center and nuclear border. Points of similar relative position, with respect to the nuclear border, formed one concentric shell. A histogram of the relative positions of the signals of probes was computed and plotted in a diagram. To be even more accurate, the histogram entry for each object point was weighted by its intensity. For statistical analysis, a list of mean 3D-RRD values for a given CT from a series of nuclei (consisting of at least 10 nuclei) was extracted. Two lists, i.e. two CTs, were compared at a time by the Mann–Whitney *U*-test.

Microarray analysis

Poly(A) mRNA was isolated from total RNA using Oligotex-dT30 Super latex beads (JSR Corporation, Tokyo, Japan), and biotinylated cRNA was synthesized according to the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix, CA, USA). The GeneChips were read using an Affymetrix GeneChip Scanner 3000 7G. These systems and data analyses were operated by the GeneChip Operating Software 1.3.

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