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Research Article

DNG1, a *Dictyostelium* homologue of tumor suppressor ING1 regulates differentiation of *Dictyostelium* cells

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Abstract. *dng1* is a *Dictyostelium* homologue of the mammalian tumor suppressor *ING* gene. DNG1 protein localizes in the nucleus, and has a highly conserved PHD finger domain found in chromatin-remodeling proteins. Both *dng1* disruption and overexpression impaired cell proliferation. In *dng1*-null cells, the progression of differentiation was delayed in a cell-density-dependent manner, and many tiny aggregates were formed. Exogenously applied cAMP pulses reversed the inhibitory effect caused by *dng1* disruption on the aggregation during early development, but formation of tiny aggregates was not restored. *dng1*-overexpressing cells acquired the ability to undergo chemotaxis to cAMP earlier and exhibited enhanced differentiation. These phenotypes were found to be coupled with altered expressions of early genes such as cAMP receptor 1 (*car1*) and contact site A (*csA)*. Furthermore, disordered histone modifications were demonstrated in *dng1*-null cells. These results suggest a regulatory role of *dng1* in the transition of cells from growth to differentiation.

Key words. *dng1; ING* family; growth; differentiation; transcription; cAMP signaling; histone modification; *Dictyostelium.*

The development and maintenance of a complex organism require precise regulation of cell proliferation and differentiation. Disorder of the regulatory system may threaten life, as realized in uncontrolled proliferation of cancer cells. However, generally, the growth/differentiation transition (GDT) occurs asynchronously in multicellular organisms, and thus analysis of the GDT mechanism at the individual cell level is difficult.

Dictyostelium discoideum is a relatively simple eukaryote, and provides an excellent system to elucidate the control mechanisms of cell growth and differentiation. *Dictyostelium* cells grow as individual amebae and proliferate by cell division as long as nutrients are available. Upon deprivation of nutrients, however, starved cells differentiate to acquire aggregation competence and aggregate to form multicellular mounds and then slugs. The slug eventually culminates to form a fruiting body consisting of a mass of spores and a supporting column of stalk cells. Several genes (*car1, dia1, dia2*) relating to cAMP signaling are induced immediately after transition of cells from growth to differentiation [1–3]. The secreted cAMP is perceived by G-protein-coupled seven-transmembrane receptors (cARs), and regulates a variety of downstream signals [4]. cAMP signaling regulates the expression of numerous genes required for development, subsequently activating several signal transduction pathways [5–7]. cAMP receptor 1 (cAR1) mainly acts on chemotaxis toward cAMP and mediates cAMP signaling during the aggregation stage [4]. The contact site A (CsA/gp80) is induced by a pulsatile, nanomolar cAMP signal and acts as an EDTA/EGTA-resistant cell adhesion molecule during the aggregation stage [8, 9]. In *Dictyostelium*, the growth and differentiation phases

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are temporally separated from each other and easily controlled by nutritional conditions. Approximately 25 % of the estimated 8000 genes in the *Dictyostelium* genome are regulated during the transition from unicellular to multicellular development [10]. So far, a large number of genes that are temporally regulated during development have been reported, and most of them participate in the progression of development [1, 2, 5, 11]. However, the mechanism of transcriptional regulation of these genes remains to be solved.

ING1 (inhibitor of growth 1) tumor suppressor was first cloned by subtractive hybridization of human breast cancer cell line cDNAs with cDNA from normal human epithelial cells, followed by an *in vivo* selection assay [12]. Several alterations of *ING1* have been reported in human cancers [13–15]. *ING* genes constitute a highly conserved gene family among a variety of eukaryotic organisms such as human, mouse, *Xenopus* and yeast. Suppression of p33ING1b expression promotes focus formation and growth *in vitro* and tumor formation *in vivo*, whereas ectopic overexpression of the protein blocks the cell cycle progression by arresting transfected cells at the G1 phase of the cell cycle in mammalian cells [12, 16]. ING1 has been shown to bind to p53, resulting in inhibition of cell growth and induction of apoptosis [17]. ING family members also bind to histone acetyltransferase (HAT) complexes and histone deacetylase (HDAC) complexes, both of which play important roles in chromatin remodeling evoked by histone acetylation and deacetylation [18–23]. Moreover, ING1 has been demonstrated to bind directly to DNA to regulate transcription [24]. We report here that an *ING1* homologue, *dng1,* in *Dictyostelium* is involved in regulation of the GDT process, presumably through transcriptional control of several genes.

Materials and methods

Cell culture and differentiation

Vegetative cells of *D. discoideum* Ax-2 were grown in shaking culture in PS medium as described elsewhere [25]. Transformed cells were grown axenically by shaking culture in PS medium containing an appropriate selection drug (blasticidin S 10 μg/ml for *dng1*-null cells, G418 50 μg/ml for *dng1*^{OE} cells, G418 20 μg/ml for *gfp-dng1* cells and s65t-GFP-expressing cells). To monitor the growth of the transformed cells in axenic conditions, they were shaken in growth medium at 22.0 °C at 150 rpm without selection drugs. Temporal changes in cell density during the exponential growth phase were determined using a hemocytometer. To monitor the growth of the cells with food bacteria, the exponential growing cells were plated with *Escherichia coli* (B/r) on LP agar plates [0.1 % Bacto peptone (Difco), 0.1 % lactose, 1.5 % agar] and 3LP agar plates [0.3 % Bacto peptone (Difco), 0.3 % lactose, 1.5 % agar] without selection drugs, and incubated for 72 h at 22 °C. The cells fed on the bacteria and formed plaques on the bacterial lawn. This was followed by measurements of the diameters of plaques formed on the bacterial lawn. To determine cell numbers in plaques formed on the bacterial lawn, cells were suspended in dissociation buffer [50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 % pronase E, 0.04 % 2, 3-dimercapto-1-propanol (BAL)] and counted using a hemocytometer. To allow cells to differentiate, they were washed with BSS as described by Mayanagi et al. [25], and settled either in a 24-well titer plate (Falcon, no. 3047) or on 1.5 % non-nutrient agar at various cell densities, and incubated at 22 °C. For preparation of total RNA, starved cells were suspended in BSS (1×10^7 cells/ml) and shaken at 150 rpm. In another experiment using cAMP pulses, pulses of 50 nM cAMP were applied to starved cell suspensions (1×10^7 cells/ml BSS) every 6 min.

Isolation of total RNA and Northern hybridization

Total RNA was isolated using a TRIzol reagent (Invitrogen). The total RNA samples (40 μg) were Northern blotted as described previously [3]. Northern hybridization was carried out at 55 °C using the [^{32}P]-labeled cDNA probe. [^{32}P]-labeled probes were prepared using the Megaprime DNA labeling system (Amersham Biosciences).

Vector constructs for knockout and overexpression of *dng1*

The vector for *dng1* knockout, pBluescript II KS(–) containing the *dng1* cDNA (SSE220), was donated by the *Dictyostelium* cDNA project in Japan (http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html). The middle position of the *dng1* open reading frame (ORF) was cut by digestion with *Eco*RI and blunted using T4 DNA polymerase (Nippongene). The vector was ligated with the blunted blasticidin resistance gene cassette containing the actin15 promoter and actin8 terminator derived from pUCBsrΔBamΔEco [26]. The vector for *s65t-gfp* expression was the pB17S plasmid containing *s65t-gfp* between the *Kpn*I and *Bam*HI in the multicloning site of pDXA-3C, donated by S. Gamboni. The pV18p-rpL11N-s65tGFP plasmid, an integrate-type vector containing the G418 resistance gene cassette was a gift from H. MacWilliams [27]. To produce the genome integrate-type A15p-s65tGFP vector, a fragment containing the actin15 promoter and *s65t-gfp* was obtained from pB17S with *Spe*I and *Xho*I, and then ligated into the pV18p-rpL11N-s65tGFP backbone after removal of V18p-*rpL11N-s65tgfp* by *Xba*I and *Xho*I. For the vector for *gfp-dng1* expression the *dng1* insert was amplified by the polymerase chain reaction (PCR) using the *Bam*HI-site-

conjugated specific primer (5'-CGGGATCCATGTATCAGGGAAAGGAA-3') and general primer M13-20. The amplified fragment was digested with *Bam*HI and *Bss*HII and ligated into a *Bam*HI-*Xho*I-digested A15p-s65tGFP vector. For the vector for *dng1* overexpression, the A15p-s65tGFP-*dng1* vector was digested with *Kpn*I and *Bam*HI to remove the s65tGFP in front of the *dng1* start codon ATG, and blunted by T4 DNA polymerase (Nippongene), followed by ligation. The sequence of *dng1* has been submitted to DDBJ with an accession number AB194261.

Preparation of the anti-DNG1 antibody

The keyhole limpet hemocyanin (KLH)-conjugated oligo-peptides (^{188}-STHNKKHKARDSLYSSSSSGN-^{209}) were prepared to immunize a rabbit. A rabbit was immunized with the oligo-peptides as described previously [25]. The IgG fraction of the polyclonal antiserum was purified using a DNG1 affinity column, which was constructed by coupling the DNG1 oligo-peptides to a HiTrap NHS-activated HP column (Amersham Biosciences).

Western blot analysis

Cells were lysed in SDS-sample buffer (2 % SDS, 60 mM Tris-HCl, 10 % glycerol, 5 % 2-mercaptoethanol) by boiling for 5 min and chilled on ice, followed by addition of 8 M urea. To detect DNG1 protein, Western blot analysis was basically performed as described previously [25]. Subcellular fractionation for isolation of nuclei was performed according to the method of Gollop and Kimmel [28] using nuclear isolation buffer (50 mM Tris-HCl, pH 7.6, 5 mM $Mg(OAc)_2$, 10 % sucrose, 2 % NP-40, 0.5 mM PMSF). For native-PAGE, cells were lysed in lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 0.2 % Triton X-100). The samples were separated on a 10 % acrylamide gel in non-denaturing buffer (90 mM Tris, 80 mM boric acid, 2.5 mM EDTA, 0.3 % Triton X-100).

Staining of cells with a DNA-specific dye, DAPI

Fixation of gfp-*dng1* cells by methanol and subsequent staining with DAPI were performed. Exponentially growing cells were fixed with absolute methanol and stained with 0.5 μg/ml DAPI in McIlvaine's buffer (0.17 M Na_2HPO_4 $2H_2O$, 13 mM citric acid, pH 7.2) for 1 h. Fluorescent images were obtained under a Nikon fluorescence microscope, using a Leica DC250 CCD camera.

Extraction of histones and acid/urea/Triton X-100 gel electrophoresis

Nuclei were harvested according to the cell fractionation method as described above, and washed twice with nuclear isolation buffer. Histones were extracted with 0.36 M H_2SO_4 overnight at 4 °C. After centrifugation, the soluble extract was recovered and precipitated with acetone. Extracted histones were dissolved in water, and an equal volume of sample loading buffer (8 M urea, 5 % mercaptoethanol, 6 % acetic acid, pyronin Y) was added. The crude histones were run on an acid/urea/Triton (AUT)-polyacrylamide gel (1 M acetic acid, 8 M urea, 1.67 % Triton X-100, 45 mM NH_3, 12 % acrylamide), basically according to the method of Yoshida et al. [29], and silver stained. Histones from calf thymus (Worthington) were used as a reference.

Results

Structural features of DNG1

Searching the *Dictyostelium* cDNA databases (http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html) [30], we found a cDNA clone containing a full-length ORF, which shares significant sequence identity with *ING* family members. The *ING* homologue in *Dictyostelium* was referred to as *dng1* (*Dictyostelium ing* 1). The deduced amino acid sequence of the *dng1* product DNG1 is shown in figure 1A. From the cDNA sequence, we assembled the genomic *dng1* and the flanking sequence using the *Dictyostelium* genomic database (http://dictybase.org/) [31]. There are three exons and two short introns in the *dng1* locus. A putative TATA box is found 212 bp upstream of the translation start codon, and a polyadenylation signal is found in 3' region of ORF (fig. 1A). Although we searched the database, we could not find other homologues of the *ING* family in *D. discoideum*. Actually, Southern analysis showed that *Dictyostelium* cells have a single copy of *dng1* (data not shown), though several other organisms have two or more *ING* genes [18, 32, 33]. The DNG1 has several putative nuclear localization signals (NLS), a coiled-coil motif in the N terminus, and a PHD finger motif in the C terminus. The PHD finger is a Cys4-His-Cys3-type zinc finger domain involved in chromatin-mediated transcriptional regulation [34]. The C terminus containing the PHD finger is highly conserved among the ING family (55–75 % identity). Unique low-complexity serine- and glycine-rich regions are also found in the middle. Alignments of the ING proteins of several species were generated using ClustalW (http://clustalw.genome.ad.jp/) (fig. 1B).

Expression and localization of DNG1

The *dng1* product is expressed at quite a low level during the entire course of development (fig. 2A). DNG1 has putative NLS, as predicted by the PSORTII program (http://psort.ims.u-tokyo.ac.jp/form2.html) in order to localize in the nucleus. To confirm this, we prepared GFP-

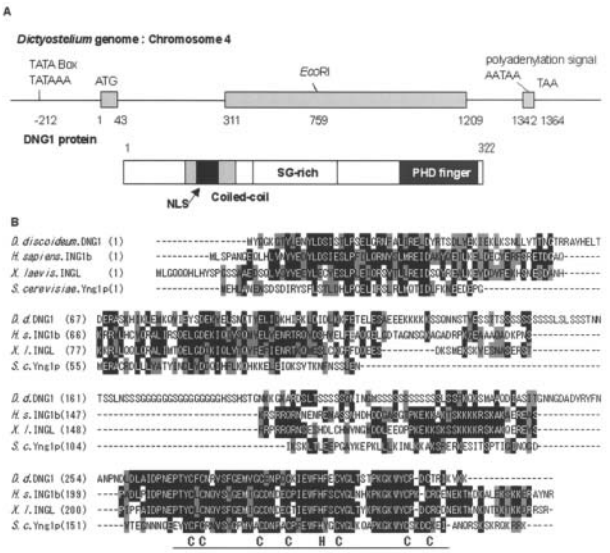

Figure 1. (A) Genomic structure of *dng1* and schematic representation of domain structures of the DNG1 protein. The *dng1* locus is composed of three exons and two introns. A putative TATA box sequence in the upstream and a polyadenylation signal sequence 3' to the ORF are shown. The DNG1 protein has several nuclear localization signals (NLS, black boxed), coiled-coil motifs (shaded) and a conserved PHD finger domain (darkly shaded). A serine- and glycine-rich region is present in the middle. (B) Amino acid sequence alignment of the ING family in several species by ClustalW. DNG1, human ING1b, *Xenopus* INGL and *Saccharomyces cerevisiae* Yng1 p were aligned [12, 18, 32]. Identical residues are indicated by black backgrounds, and conservative substitution by gray backgrounds. Gaps introduced to optimize the alignment are marked by dashes. C and H indicate the conserved Zn^{2+}-chlating cysteine and histidine in the PHD finger domain.

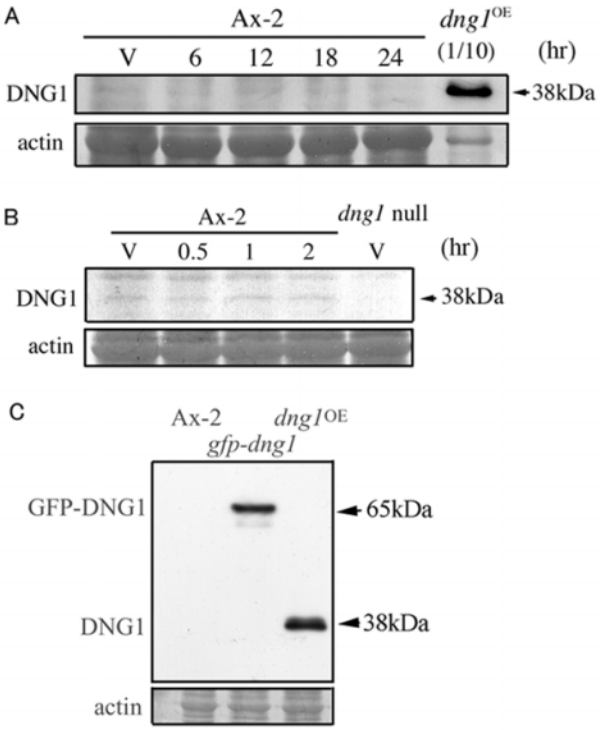

Figure 2. Expression patterns of DNG1 protein during development of *D. discoideum* Ax-2. (A) Ax-2 cells grown axenically in growth medium were harvested at the exponential growth phase ($3–5 \times 10^6$ cells/ml), washed and allowed to differentiate on 1.5 % non-nutrient agar. Protein samples were prepared at the indicated times, separated by 10 % SDS-PAGE and blotted. Blots were immuno-detected using the anti-DNG1 antibody. A one-tenth-diluted *dng1*^{OE} sample was loaded to detect the DNG1 band of 38 kDa. (B) Protein samples were prepared from parental Ax-2 cells and *dng1*-null cells and analyzed by Western blotting using the anti-DNG1 antibody. The films were exposed for a long time (about ten-times longer exposure relative to C). (C) Protein samples were prepared from parental Ax-2 cells, *dng1*^{OE} cells and *gfp-dng1* cells, followed by Western blotting using the anti-DNG1 antibody. The amount of actin stained with Coomassie Brilliant Blue is presented as a loading control.

DNG1 fusion protein and monitored its behavior. GFP fluorescence was found to be exclusively localized in the nucleus throughout development (fig. 3A, B). This was confirmed by Western blot analysis using isolated subcellular fractions (fig. 3C). DNG1 protein was detected exclusively in the nuclear fraction, while HSC90 (cytosolic Hsp90 in *D. discoideum*) [35] was predominantly detected in the cytosolic fraction.

dng1-null cells and *dng1*-overexpressing cells exhibit slow growth

To analyze *dng1* function, the *dng1* gene was disrupted by homologous recombination or overexpressed using the *dng1* cDNA constitutively expressed under the control of the actin 15 promoter. The amount of DNG1 protein was confirmed by Western blot analysis (fig. 2B, C). When these transformants and parental-type Ax-2 cells were incubated on the bacterial lawn (LP agar plate and 3LP agar plate), both *dng1*-null and *dng1*-overexpressing cells (*dng1*^{OE} cells) exhibited slower growth compared to Ax-2 cells (fig. 4, table 1). A similar result was obtained when the cells were incubated in *E. coli* suspension by shaking culture (data not shown). However, such a marked effect on growth was not detected in axenic shaking culture (data not shown).

Altered *dng1* expression affects development of starved cells

In addition to the effect of *dng1* expression on cell growth, altered *dng1* expression was found to evoke interesting phenotypes during the differentiation phase after starvation. Under submerged conditions in BSS, *dng1*-null cells exhibited delayed aggregation compared to Ax-2 cells, particularly at a low cell density (5×10^4 cells/cm^2), and formed numerous tiny aggregates. Although the delayed aggregation was scarcely noticed when starved *dng1*-

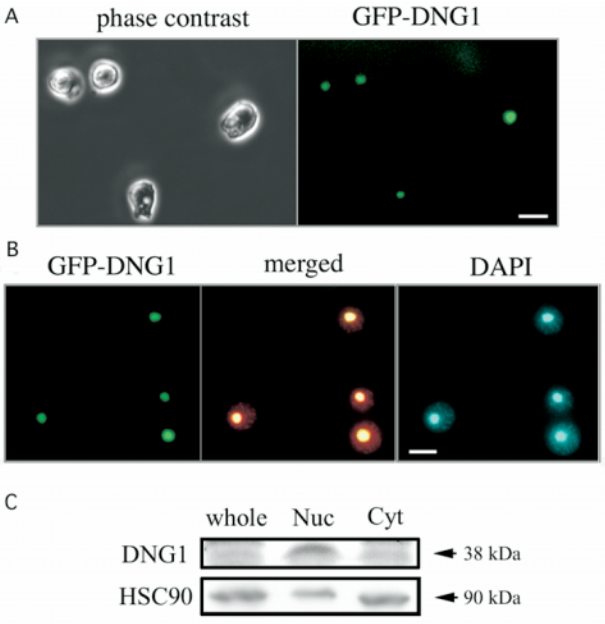

Figure 3. Nuclear localization of DNG1. (A) Living GFP-DNG1 cells at the growth phase were investigated using phase contrast (left) and fluorescent (right) microscopes. (B) Growing GFP-DNG1 cells were fixed in methanol and then stained with DAPI. The GFP signal of DNG1 completely corresponds to nuclear staining by DAPI. The DAPI-stained signal was artificially converted to red color to produce the merged figure (middle). Bar, 10 μm. (C) Western blot analysis of nuclear fraction (Nuc) and cytosolic fraction (Cyt) using the anti-DNG1 antibody and anti-HSC90 antibody. Exponentially growing Ax-2 cells were lysed and fractionated as described in Materials and methods. DNG1 protein was detected only in the nuclear fraction. HSC90 was predominantly found in the cytosolic fraction.

Table1. Growth of *dng1*-null, *dng1*^{OE} and parental Ax-2 cells on the bacterial lawn.

Medium	Ax-2	*dng1*-null	*dng1*^{OE}
Plaque diameter (mm, mean ± SD)			
LP	6.38 ± 1.26	3.25 ± 0.80*	4.33 ± 1.91*
3 LP	4.74 ± 1.24	2.50 ± 0.58*	3.59 ± 1.22*
Cell numbers in plaques (×10^4 cells/plaque, mean ± SD)			
LP	13.01 ± 2.23	2.69 ± 0.60*	3.52 ± 0.89*
3 LP	14.92 ± 2.44	3.73 ± 1.13*	3.33 ± 1.16*

To monitor the growth of the cells with food bacteria, exponentially growing cells were plated with *E. coli* (B/r) on LP and 3LP agar plates without selection drugs, and incubated for 72 h at 22 °C. The cells fed on the bacteria and formed plaques on the bacterial lawn. This was followed by measurements of the diameters of plaques formed on the bacterial lawn. The cell numbers in plaques were counted according to the procedure described in Materials and methods.

* *dng1*-null and *dng1*^{OE} cells are significantly different from those of parental Ax-2 ($p < 0.001$).

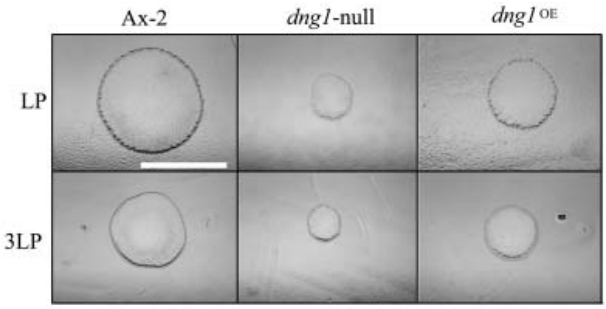

Figure 4. Growth of *dng1*-null, *dng1*^{OE} and parental Ax-2 cells on the bacterial lawn. Exponentially growing cells were plated with *E. coli* on LP and 3LP agar plates and incubated at 22.0 °C without selection drugs. Cells grew by feeding on bacteria and formed plaques. The diameters of the plaques were measured after 72 h incubation. Representative results of three independent experiments are shown. Bar, 5 mm.

null cells were incubated at a high cell density (2.3×10^5 cells/cm^2), the phenotype of forming tiny aggregates was retained (fig. 5A). On non-nutrient agar, starved *dng1*-null cells showed slightly slower aggregation than Ax-2 cells and formed a number of tiny aggregates at a low cell density (1.5×10^6 cells/cm^2), as was the case for the submerged conditions (fig. 5B). At a high cell density (3×10^6 cells/cm^2), however, the delay was not observed (fig. 5B). In other words, starved *dng1*-null cells formed more aggregation centers compared to parental Ax-2 cells, resulting in a higher number of tiny migrating slugs. Incidentally, *dng1*-null cells completed formation of tiny fruiting bodies 3–4 h faster than Ax-2 cells.

On the other hand, starved *dng1*^{OE} cells exhibited more rapid aggregation both under submerged conditions and on non-nutrient agar. They acquired aggregation competence 4 h after starvation and then formed aggregation streams earlier than Ax-2 cells under submerged conditions (fig. 6A). On non-nutrient agar, *dng1*^{OE} cells formed aggregation streams that were sometimes subdivided to smaller mounds. Each mound began to culminate about 18 h after starvation, and completed formation of fruiting bodies earlier than parental Ax-2 cells (fig. 6B).

Effect of caffeine on developmental phenotypes of *dng1*-null and *dng1*^{OE} cells

The results presented above suggest the involvement of cell-to-cell communication, as realized by cAMP signaling, in developmental behaviors of *dng1* transformants. Caffeine, an inhibitor of adenylyl cyclase activation, has been demonstrated to inhibit cAMP signaling in *Dictyostelium* [36]. Therefore, we examined the effects of caffeine on the *dng1* transformants and parental Ax-2 cells. Ax-2 cells suffered severe developmental defects in the presence of 5 mM caffeine, and hardly aggregated at 7.5 mM caffeine (fig. 7). As was expected, most *dng1*-

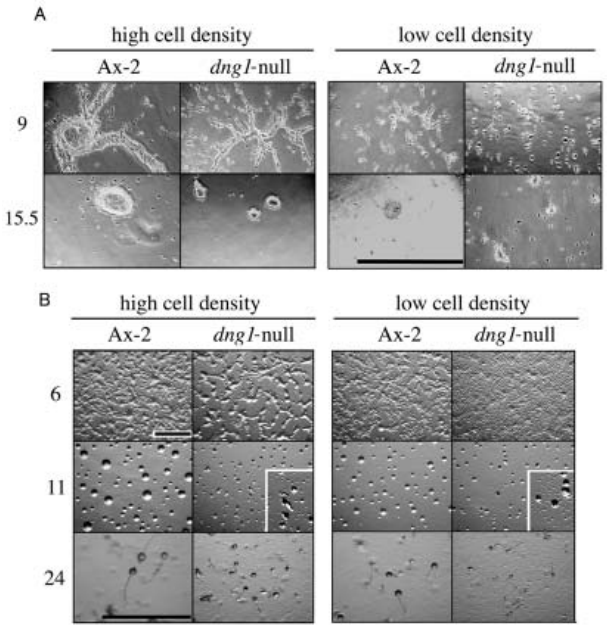

Figure 5. Morphogenesis of starved *dng1*-null and Ax-2 cells under submerged conditions and on 1.5 % non-nutrient agar. (A) *dng1*-null and Ax-2 cells were separately harvested at the exponential growth phase, washed twice in BSS and plated in a 24-well titer plate at the indicated high (2.3×10^5 cells/cm^2) or low (5×10^4 cells/cm^2) cell densities. Bar, 500 µm. (B) *dng1*-null and Ax-2 cells were separately harvested at the exponential growth phase, washed twice in BSS and plated on 1.5 % non-nutrient agar at a high (3×10^6 cells/cm^2) or a low (1.5×10^6 cells/cm^2) cell density. Bar, 1 mm.

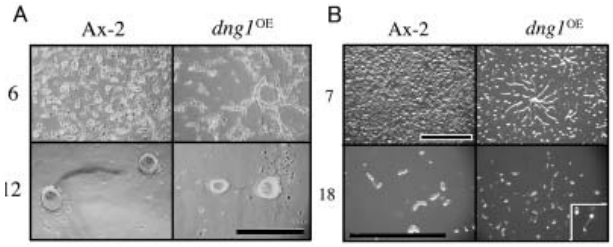

Figure 6. Morphogenesis of starved *dng1*^{OE} and Ax-2 cells under either submerged conditions or on 1.5 % non-nutrient agar. (A) *dng1*^{OE} and Ax-2 cells were separately harvested at the exponential growth phase, washed twice in BSS and plated in a 24-well titer plate at a cell density of 5×10^5 cells/cm^2. Bar, 250 µm. (B) *dng1*^{OE} and Ax-2 cells were each harvested at the exponential growth stage, washed twice in BSS and plated on 1.5 % non-nutrient agar at a cell density of 3×10^6 cells/cm^2. Bar, 1 mm.

null cells remained as non-aggregated single cells in the presence of 5 mM caffeine, though a limited number of cells locally aggregated to form standing slugs (fig. 7). In contrast, *dng1*^{OE} cells showed much stronger resistance to caffeine. They aggregated and formed migrating slugs at 5 mM and were able to form aggregates even in the presence of 7.5 mM caffeine (fig. 7).

dng1 affects early developmental gene expression

To examine if *dng1* expression exerts transcriptional control of early developmental genes such as *car1* and *csA*, total RNA was isolated from cells at various developmental stages, followed by Northern blotting. We found that while *dng1* expression enhanced induction of *car1* expression, *car1* expression was impaired and reduced in *dng1*-null cells (fig. 8). *csA* expression was also decreased in *dng1*-null cells and slightly enhanced in *dng1*^{OE} cells, as was the case for *car1* expression (fig. 8).

DNG1 is a component of large complexes and affects histone modification

DNG1 is predicted to function as a transcriptional regulator interacting with transcriptional co-factors such as HAT and HDAC complexes. We examined how DNG1 is present in *Dictyostelium* cells using native-PAGE Western analysis. We found that DNG1 protein forms several complexes, one of which is large (> 200 kDa), and that the status of the complex changes during the transition from growth to differentiation (fig. 9A). DNG1 also seemed to play a role in transcriptional control via interaction with other proteins, though the molecular partner(s) remain to be elucidated. Furthermore, we performed AUT-PAGE to investigate the status of histone modification. AUT gel electrophoresis allowed separation of cellular histones (H1, H2A, H2B, H3 and H4) with modifications of differing extent due to the slower migration rates of the acetylated or phosphorylated species [29]. Figure 9B shows profiles of histones extracted from Ax-2 and *dng1*-null cells at the growth and the differentiation phase. In Ax-2 cells, the modification level of histone H2B appeared to be increased in response to cell differentiation. *dng1*-null cells exhibited a disordered change of histone H2B modification. In addition, proper histone H1 and H3 modifications were also impaired in *dng1*-null cells, suggesting the importance of DNG1 in transcriptional regulation via histone modification.

Application of cAMP pulses overcomes the aggregative defect observed in *dng1*-null cells

The secretion of periodic cAMP pulses is known to be required for cell aggregation. An exogenous cAMP pulse was reported to overcome the defects of several aggregation-deficient mutants. When cAMP pulses were exogenously added to *dng1*-null cells, they restored almost normal *car1* and *csA* expression (fig. 10A). The cAMP pulses also countered the aggregation retardation of *dng1*-null cells, as shown in figure 10B. Taking these together with results shown in figures 5, 7 and 8, *dng1*-null cells clearly have a defect in cAMP signaling. However, the developmental feature of *dng1*-null cells

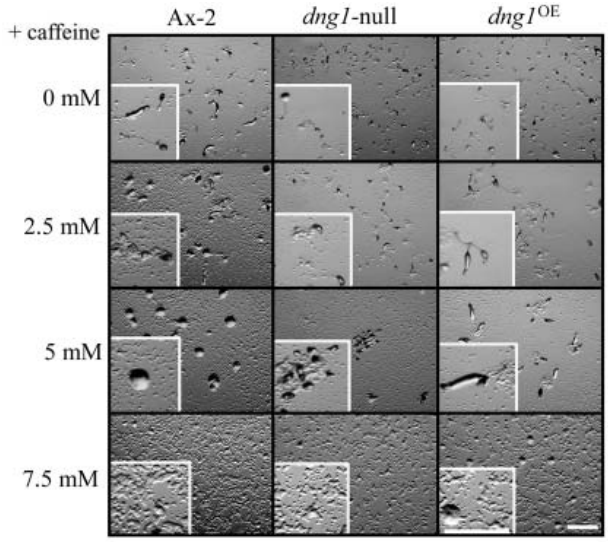

Figure 7. Effects of caffeine on development of starved *dng1*-null, *dng1*^{OE} and Ax-2 cells. The three kinds of cell were separately developed on 1.5 % non-nutrient agar containing the indicated concentrations of caffeine at a cell density of 1.5×10^6 cells/cm^2, followed by incubation for 24 h at 22 °C. Bar, 1 mm.

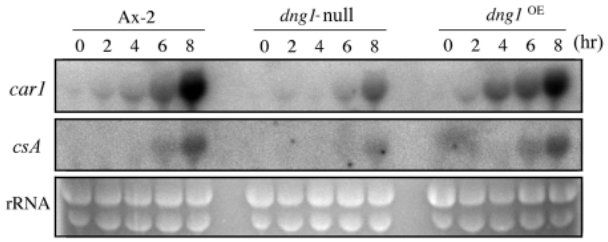

Figure 8. Expression patterns of *car1* and *csA* in cells with different amounts of DNG1. Total RNA (40 µg/lane) was prepared from Ax-2, *dng1*-null and *dng1*^{OE} cells at the indicated times of suspension culture in BSS. Total RNA was analyzed using the [^{32}P]-labeled cDNA probes for *car1* and *csA*.

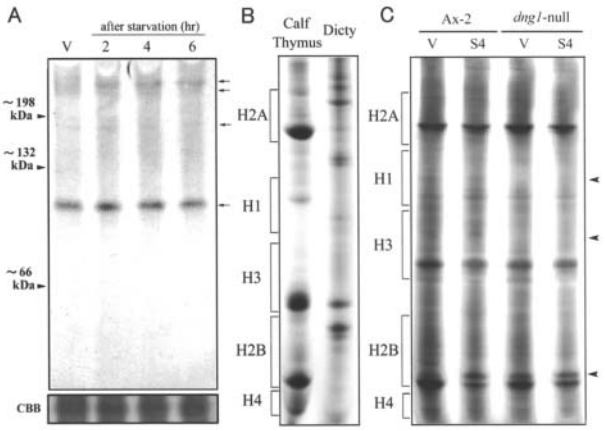

Figure 9. (A) Native-PAGE of DNG1 protein in *Dictyostelium* cells. Non-denatured samples were prepared from *dng1*^{OE} cells at the indicated times of suspension culture in BSS. The samples were separated on native-PAGE and blotted to membranes. Blots were immuno-detected using the anti-DNG1 antibody. Arrowheads indicate approximate molecular weights estimated from standard BSA. Arrows indicate several species of complex containing DNG1. The band (approximate 100 kDa) stained with Coomassie Brilliant Blue is presented as a loading control. (B) Histone separation on AUT gels. Histones were extracted from Ax-2 cells and the crude histones electrophoretically separated on a 14 % AUT gel, and silver stained. Histones from calf thymus were used as a reference. (C) Histone modification during development. Histones were extracted from Ax-2 cells and *dng1*-null cells during the growth phase and the differentiation phase (4 h shaking culture after starvation), respectively. The extracted crude histones were electrophoretically separated on a 12 % AUT gel, and silver stained. Arrowheads indicate the altered patterns of modified histones.

as characterized by tiny-aggregate formation was never restored by the cAMP pulses, while parental Ax-2 cells formed somewhat larger aggregates compared to non-pulsed cells.

Effect of conditioned medium on development of Ax-2 cells

Starving cells have been demonstrated to secrete several factors such as counting factor (CF) that regulate the size of cell aggregation [37, 38]. To study whether secreted factors are involved in the aggregation size of *dng1*-null cells, conditioned starvation medium (CSM) was prepared as the supernatant of 6 and 12 h-starved *dng1*-null, *dng1*^{OE} and Ax-2 cells, their effects on the development of Ax-2 cells examined to identify what causes small aggregates in *dng1*-null cells. CSM prepared from *dng1*-null cells had no significant effect on the development of Ax-2 cells compared with that prepared from Ax-2 cells, while CSM prepared from *dng1*^{OE} cells only enhanced the progression of morphogenesis including aggregation (data not shown). These results seem to indicate that tiny-aggregate formation as observed in *dng1*-null cells may not be due to secreted factors like CF.

Cell autonomous defect of *dng1*-null cells

We performed the synergy experiment to examine further the character of the cell-autonomous defect in *dng1*-null cells. When starved *dng1*-null and Ax-2 cells were mixed at various ratios and co-developed, apparent synergy was not observed between them. The aggregation size was reduced as the relative ratio of *dng1*-null cells increased (data not shown). In another experiment, a small number of starved s65t-GFP-expressing *dng1*-null cells were mixed with Ax-2 cells and incubated, to trace the distribution of *dng1*-null cells in aggregation streams and subsequent multicellular structures. Although *dng1*-

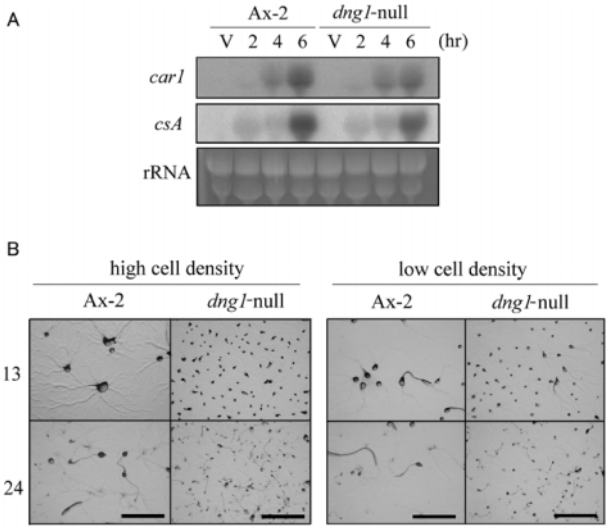

Figure 10. Effects of exogenously applied cAMP pulses on *car1* and *csA* expression, and on morphogenesis. *dng1*-null and Ax-2 cells were separately starved by washing twice in BSS and allowed to differentiate in suspension culture. Cell suspensions (1×10^7 cells/ml) were supplemented with exogenous 50 nM cAMP pulses every 6 min. (A) Total RNA (40 μg/lane) was prepared at the indicated times and analyzed using the [^{32}P]-labeled cDNA probe. (B) After 6 h incubation with cAMP pulses, cells were plated on 1.5% non-nutrient agar at a high (3×10^6 cells/cm^2) or low (1.5×10^6 cells/cm^2) cell density and incubated at 22 °C. The numbers indicate the total periods after starvation. Bar, 1 mm.

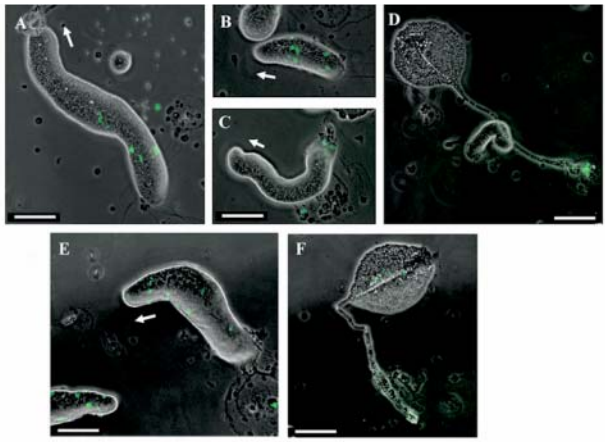

Figure 11. Behavior of *dng1*-null cells in chimeric development. GFP-expressing *dng1*-null cells and parental Ax-2 cells were mixed at a ratio of 1 : 99 and co-developed for 18 h (A, B, C) and 24 h (D). Phase contrast and fluorescent (GFP) images were obtained by an epi-illumination phase contrast fluorescence microscope, and merged. GFP-expressing *dng1*-null cells were predominantly located in the posterior region of migrating slugs (A, B), and sometimes in the rearguard region (C). Eventually, GFP-expressing *dng1*-null cells were hardly detected in the spore mass of a fruiting body (D). As a control, when GFP-expressing Ax-2 cells and Ax-2 cells were co-developed on agar at the ratio of 1 : 99 for 18 h (E) and 24 h (F), uneven distribution of GFP fluorescence was not observed (E, F). Arrows indicate the direction of slug migration. Bar, 100 μm.

null cells seemed to be uniformly distributed during the aggregation stage, they began to localize in the posterior region of a migrating slug (fig. 11A–C), and were eventually left behind in the rearguard region (fig. 11C). Thus *dng1*-null cells were predominantly located in the basal disc of a culminating cell mass, and were scarcely detected in spores (fig. 11D). These results confirmed that *dng1*-null cells have an apparently cell-autonomous defect. The abnormal distribution of *dng1*-null cells may also be derived from the defective responce to cAMP in the organization of the migrating slugs.

Discussion

We have reported the structure and function of an *ING1* homologue *dng1* in *D. discoideum*. The deduced amino acid sequence shares significant identity with *ING* family members, and has a highly conserved PHD finger domain. The PHD finger domain has been found in many proteins relating to chromatin regulation and transcriptional control [34]. Recent studies have demonstrated that the PHD finger of ING2 functions as a nuclear phosphoinositide receptor and regulates the ability of ING2 to activate p53-dependent apoptotic pathways [39–41]. Actually, ING1b and ING2 protein repress the activity of the α-fetoprotein promoter but stimulate p21 promoter activity in human cultured cells [24]. As presented in this paper, *dng1* mRNA and DNG1 protein are expressed at quite a low level during development, and DNG1 is exclusively localized in the nucleus, as is the case for other ING members. Altered *ING* expression is known to greatly affect cell growth in several cell lines [17, 18, 42]. In vertebrate cells, ING1 plays important roles in growth regulation and in tumor suppression in a p53-dependent and -independent pathway [16, 38, 39]. Although the *Dictyostelium* genome project has been almost completed, no gene defined as a counterpart of p53 has yet been found in *Dictyostelium*. Of interest would be to know if there is a molecule with a p53-like property in *Dictyostelium*. In the case of *dng1*-null and *dng1*^{OE} cells, their growth was considerably impaired compared to parental Ax-2 cells. This suggests that DNG1 also has an important function in growth control of *Dictyostelium* cells. Furthermore, we found that alterations in *dng1* expression markedly affect the differentiation processes of starved cells: knockout of *dng1* caused a decrease in cAMP signaling activity, leading to delayed aggregation, while *dng1*-overexpression enhanced cell aggregation. Of interest to note here is that delayed aggregation of *dng1*-null cells was reversed by culture of starved cells at a high cell density, and also mostly reversed by application

of cAMP pulses in low cell-density cultures, recovering *car1* and *csA* expression. In contrast to *dng1*-null cells, *dng1*^{OE} cells showed more rapid aggregation than Ax-2 cells, and were able to aggregate even on agar containing caffeine. The expression of *car1* and *csA* was also found to be slightly enhanced in *dng1*^{OE} cells. This suggests that DNG1 takes a part in programmed differentiation of starving cells through the regulation of gene expression, though DNG1 is not essential for differentiation.

In *dng1*-null cells, cell aggregation was retarded, and small aggregates were formed. Since the *dng1*-null phenotype could not be mimicked by developing Ax-2 cells in the presence of *dng1*-null CSM, the small aggregates of *dng1*-null cells may not be due to oversecretion of CF. Moreover, apparent synergy was not observed in a mixed culture of starved *dng1*-null cells and Ax-2 cells. These results suggest that *dng1*-null cells still possess a cell-autonomous defect in the cAMP signal relay and/or cell adhesion, though the expression of *car1* and *csA* was partially rescued by cAMP pulses. The cell-autonomous defects probably cause the reduction of aggregation territory. Moreover, in co-development with parental Ax-2 cells, *dng1*-null cells were located in the posterior rearguard region of migrating slugs because *dng1*-null cells were left behind due to the cell-autonomous defects.

ING proteins have been shown to interact with several HAT or HDAC complexes and regulate acetylation of histones [18, 19, 21], leading to a change in chromatin conformation and modulation of global transcriptional activity. Most HATs have proven to be transcriptional adaptors or co-activators, whereas HDACs generally act as co-repressors. We performed native-PAGE and demonstrated that DNG1 protein forms large molecular complexes in *Dictyostelium* cells, suggesting a likely function of DNG1 protein in the large complexes as transcriptional co-factors. In fact, *dng1*-null cells exhibited some defects of the control of histone modification, probably by acetylation, which may affect the transcriptional regulation during the growth/differentiation transition. *Dictyostelium* development consists of a series of synchronous, coordinated morphological and physiological changes, accompanying drastic changes of expressed genes, such as *car1* and *csA* induction during early differentiation. Based on these facts, DNG1 protein is possibly closely involved in chromatin-mediated transcriptional control over the developmental program.

It is believed that relatively primitive eukaryotes such as *Dictyostelium* do not have a system of DNA methylation-mediated gene regulation. In fact, methylated cytosine is not found in genomic DNA and never detected in primitive organisms, at least at the resolution level of HPLC analyses [43–46]. Rather, transcriptional control by histone modification may be crucial in the dynamic transition of gene expression during *Dictyostelium* development. In relation to this, pretreatment of *Dictyostelium* cells with butyrate induces hyperacetylation of histone H4 and enhances differentiation [47], and the effect of butyrate is somewhat similar to the *dng1*-overexpressed phenotype. These findings suggest the importance of epigenetic transcriptional regulation in the transition of cells from growth to differentiation.

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