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## RESEARCH ARTICLE

# Different CHD chromatin remodelers are required for expression of distinct gene sets and specific stages during development of *Dictyostelium discoideum*

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## ABSTRACT

Control of chromatin structure is crucial for multicellular development and regulation of cell differentiation. The CHD (chromodomain-helicase-DNA binding) protein family is one of the major ATP-dependent, chromatin remodeling factors that regulate nucleosome positioning and access of transcription factors and RNA polymerase to the eukaryotic genome. There are three mammalian CHD subfamilies and their impaired functions are associated with several human diseases. Here, we identify three CHD orthologs (ChdA, ChdB and ChdC) in *Dictyostelium discoideum*. These CHDs are expressed throughout development, but with unique patterns. Null mutants lacking each CHD have distinct phenotypes that reflect their expression patterns and suggest functional specificity. Accordingly, using genome-wide (RNA-seq) transcriptome profiling for each null strain, we show that the different CHDs regulate distinct gene sets during both growth and development. ChdC is an apparent ortholog of the mammalian Class III CHD group that is associated with the human CHARGE syndrome, and GO analyses of aberrant gene expression in *chdC* nulls suggest defects in both cell-autonomous and non-autonomous signaling, which have been confirmed through analyses of *chdC* nulls developed in pure populations or with low levels of wild-type cells. This study provides novel insight into the broad function of CHDs in the regulation development and disease, through chromatin-mediated changes in directed gene expression.

KEY WORDS: SNF2, Transcriptome profiling, RNA-seq, Chemotaxis, Growth, Differentiation

## INTRODUCTION

The packaging of DNA into chromatin physically limits access of transcription factors and other elements of the transcription machinery to their target genes. Throughout development, changes in chromatin organization are exploited to control gene expression. In particular, alterations in nucleosome positioning along genomic DNA, termed chromatin remodeling, impact transcriptional activity, both positively and negatively (Ho and Crabtree, 2010; Euskirchen et al., 2012). Importantly, as nucleosome positions are preserved during DNA replication, inherited differences in positioning can alter multicellular development or cellular properties during disease states.

ATP-dependent chromatin remodeling proteins use the energy of ATP hydrolysis to physically change the interaction between DNA and nucleosomes, which can promote or limit DNA access to a variety of regulators (Hopfner et al., 2012). The archetypal ATP-dependent chromatin remodeler was discovered in yeast where mutations in a single yeast gene, designated *SWI2/SNF2*, lead to aberrant mating-type switching (*swi2*) (Breeden and Nasmyth, 1987) and glucose repression (*snf2*) (Abrams et al., 1986). *SWI2/SNF2* has a DNA-dependent (helicase-like) ATPase that is part of a large multiprotein complex. The *Drosophila* ortholog of SWI2/SNF2, Brahma (Brm), regulates cell fate by acting as a transcriptional co-activator of homeotic gene expression in the Antennapedia and Bithorax complexes (Tamkun et al., 1992), and the mammalian ortholog BRG1 (SMARCA4 – Mouse Genome Informatics), Brahma-related gene 1, is implicated in T-cell development, ES cell differentiation and tumor suppression (Hargreaves and Crabtree, 2011).

Other chromatin remodeling families have been identified that share the ATPase motif, but differ in other protein domains (Clapier and Cairns, 2009; Eisen et al., 1995; Ryan and Owen-Hughes, 2011). These include ISWI (imitation switch), INO80 (inositol requiring 80) and CHD (chromodomain, helicase, DNA binding) proteins (Ho and Crabtree, 2010). The first member of the CHD family was identified in mice (Delmas et al., 1993), but CHD family members were subsequently described in a variety of eukaryotic organisms, including humans, *Drosophila*, *Saccharomyces cerevisiae*, *C. elegans* and *Arabidopsis* (Flanagan et al., 2007). The human CHD family comprises nine members, CHD1-9, divided into three subfamilies – CHD1-2 (class I), CHD3-5 (class II) and CHD6-9 (class III) – based upon their chromodomain types and additional motif features (Yap and Zhou, 2011).

Functionally, the CHD proteins play important roles in the development of multicellular organisms. CHD1 is essential for maintaining pluripopotency of mouse ES cells (Gaspar-Maia et al., 2009), and *Drosophila Chd1* nulls are both male and female sterile (McDaniel et al., 2008). Loss of *Chd2* in mice causes embryonic lethality (Marfella et al., 2006a) and conditional inactivation of *CHD4* in hematopoietic cells impairs their differentiation and development (Williams et al., 2004). Haploinsufficient mutations of *CHD7* are observed in 60-80% of cases of CHARGE syndrome (Janssen et al., 2012; Lalani et al., 2006; Layman et al., 2010; Zentner et al., 2010), a severe developmental disorder in humans characterized by coloboma of the eye, heart defects, atresia of the choanae, retardation of growth, and genital and ear abnormalities (Pagon et al., 1981). *Chd7^{+/−}* mice recapitulate some of the human symptoms, and *Chd7^{−/−}* mice are embryonic lethal (E10.5) (Bosman

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et al., 2005; Hurd et al., 2007). Wild-type CHD7 can direct nucleosome sliding *in vitro*, and CHD7 proteins that recapitulate CHARGE mutations are significantly impaired in this activity (Bouazoune and Kingston, 2012). CHD7 is suggested to function on tissue-specific genes that regulate development and differentiation (Schnetz et al., 2009; Schnetz et al., 2010). CHD8 also has an important role in development, is highly expressed during early embryogenesis and appears to modulate WNT/β-catenin signaling through interaction and downregulation of response genes (Nishiyama et al., 2012; Thompson et al., 2008). Other CHD functions are associated with human diseases, including some neuroblastomas (CHD5), Hodgkin's lymphoma (CHD3) (Lemos et al., 2003) and small-cell lung cancers (CHD7) (Pleasance et al., 2010).

It is not yet clear how the different CHDs may cause these multiple, diverse and distinct patterns of developmental deficiencies. Broad investigations of the developmental targets of the individual CHD proteins and their effect on cell behavior are required to establish the connectivity of CHD function with gene expression, developmental phenotypes and disease states. We have now identified the CHD orthologs of *Dictyostelium* and associate loss-of-function mutations with distinct developmental phenotypes and altered gene expression profiles.

*Dictyostelium* grow as single-celled organisms, in which cellular growth and division are dependent on an adequate food supply (McMains et al., 2008). Nutrient depletion triggers a developmental program to ultimately form a terminally differentiated, multicellular structure: the fruiting body. Development occurs in distinct stages as cells first sense starvation and high population density, and then aggregate via a chemotactic response to secreted cAMP signals to form the initial multicellular organism. These multicellular mounds then differentiate into patterned prespore and prestalk cell populations, before terminal differentiation into structures comprising mature spore and stalk cells (Kessin, 2001).

Using genome-wide transcript profiling (RNA-seq) during growth and development to assay gene expression in *Dictyostelium* (Loomis and Shaulsky, 2011; Parikh et al., 2010) that individually lack the different CHD family members, we correlate altered transcription profiles with the developmental phenotypes of the different *chd* nulls. We conclude that the different CHDs act to control diverse aspects of *Dictyostelium* development, perhaps by targeting distinct sets of genetic loci.

ChdC in *Dictyostelium* is an apparent ortholog of the mammalian Class III CHD group that is associated with the human CHARGE syndrome. Similar to *CHD7* mutations in humans (Zentner et al., 2010), *chdC* nulls have the strongest phenotype in *Dictyostelium*, with impaired growth, chemotaxis, cell differentiation and developmental arrest. Analyses following transcriptome profiling indicate defects in both cell-autonomous and non-autonomous functions, phenotypes confirmed by chimeric development of *chdC* nulls with wild-type cells. These data yield novel insight into the broad function of CHDs, with particular relevance to the Class III CHD group and their association with highly severe genetic syndromes in humans.

## RESULTS
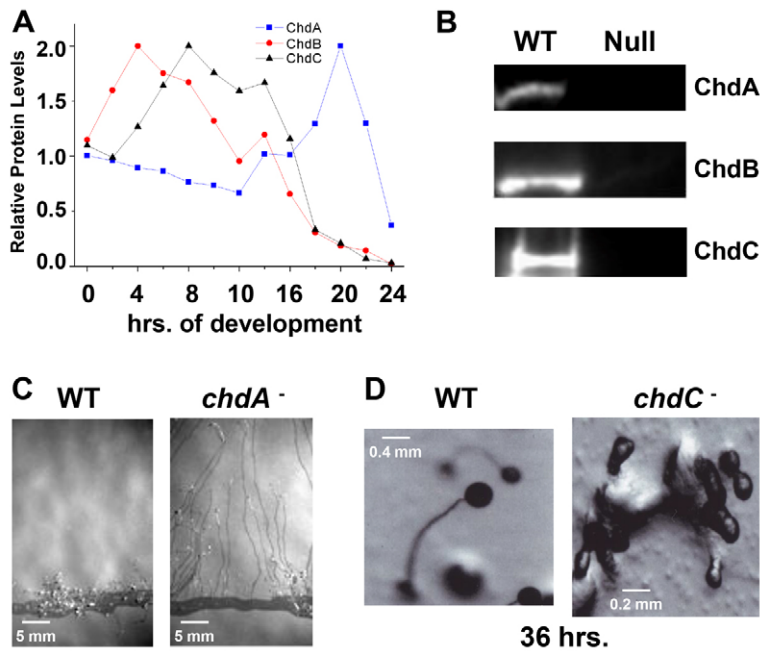### *Dictyostelium* possess three CHD proteins
We initiated separate insertional (REMI) mutagenic screens (Kuspa, 2006; Artemenko et al., 2011) to identify *Dictyostelium* strains that exhibited defects in chemotaxis to cAMP or that arrested development. An identical CHD-encoding gene was identified in these distinct screens as a novel genetic modifier of both chemotaxis

and development. Extended searches for related sequences in the *Dictyostelium* genome identified two additional CHD proteins (supplementary material Fig. S1). We have termed the *Dictyostelium* CHD genes *ChdA* (DDB_G0284171), *ChdB* (DDB_G0280705) and *ChdC* (DDB_G0293012); *ChdC* was the gene found in the original mutagenic screens. Our sequence analyses also identified related SWI2/SNF2-type genes (Ryan and Owen-Hughes, 2011) in *Dictyostelium*, encoding members of the bona fide SWI2/SNF2 (*snf2*; DDB_G0271052 and DDB_G0285205), ISWI (*isw*; DDB_G0292948), SWR (*swr1*; DDB_G0267638) and INO80 (*ino80*; DDB_G0292358) chromatin remodeling families (see supplementary material Figs S1, S2). All of the encoded proteins possess the DNA-dependent ATPase domain and a DNA-binding motif, but differ in overall organization (supplementary material Fig. S2); the tandem chromodomains are a defining moiety for the CHDs (Blus et al., 2011). More distantly related SWI2/SNF2 members in *Dictyostelium* were also noted, including the Mot1 (modifier of transcription) and Rad54 families (supplementary material Fig. S1).

*Dictyostelium* ChdA shows the strongest similarity to human CHD subfamily I, clustering with human CHD1/2 and other species Chd1-subtypes through the entire protein sequence (Ryan et al., 2011), including the C-terminal DUF4208 (supplementary material Figs S1, S2). *Dictyostelium* ChdB possesses weak C-terminal CHDCT2 domains, which are generally characteristic of CHD subfamily II (Ryan and Owen-Hughes, 2011); however, ChdB lacks the N-terminal PHD finger present in most subfamily II members and globally aligns better with human CHD6-9 proteins (supplementary material Fig. S1). ChdC (DDB_G0293012) is the largest *Dictyostelium* CHD, and clusters most strongly with the CHD6-9, subfamily III group (supplementary material Figs S1, S2). Although ChdC has C-terminal SANT and SLIDE DBD motifs characteristic of other CHD III members (Ryan and Owen-Hughes, 2011), it lacks the conserved BRK site. Motif prediction in ChdC is complicated by the large number of homopolymeric residues embedded in its long C-terminal region. In addition, although BRK domains are of unknown function, they appear to be unique to the metazoa; thus, although the yeast SWI2/SNF2 and metazoan Brm/BRG are considered to be orthologs and functionally equivalent, only Brm/BRG proteins possess BRK domains (Ryan and Owen-Hughes, 2011). ChdC may uniquely possess a SUMO-like amino acid sequence of unknown function. Collectively, the individual CHDs of *Dictyostelium* would seem to embody different CHD types.

### Developmental expression of ChdA, ChdB and ChdC
We examined developmental protein expression patterns of each CHD by immunoblotting, using protein-specific antibodies (Fig. 1A). Each CHD is detected during growth and the early stages of development, but they exhibit large expression differences following multicellular aggregation. ChdA has low relative expression during aggregation and early stages of multicellular development, but has increased abundance during terminal differentiation, after 16 hours. By contrast, ChdB shows peak abundance at 4 hours of development, the mid-stage of cellular aggregation (Fig. 1A). ChdB abundance then declines and is undetectable during late stages of development. Finally, ChdC shows peak abundance between 8 and 10 hours of development, as cells complete aggregate formation and initiate multicellular development. Again, as seen for ChdB, ChdC is undetected during late stages of development; thus, ChdA predominates at terminal differentiation.

Fig. 1. The three CHD proteins of *Dictyostelium* have distinct developmental roles. (A) Whole-cell extracts were prepared from developing *Dictyostelium* at 2-hour intervals and ChdA, ChdB and ChdC proteins levels measured by immunoblot assay. Quantified data are expressed as normalized protein levels relative to expression at 0 hour. (B) Whole-cell extracts were prepared from growing wild-type, *chdA*-null, *chdB*-null and *chdC*-null cells, and ChdA, ChdB and ChdC proteins expression measured by immunoblot assay. (C) Wild-type and *chdA*-null cells were plated for development at the edge of nitrocellulose membranes and pseudoplasmodia migration assayed by exposure to a directional light source. (D) Wild-type and *chdC*-null cells were terminally developed. *chdC* nulls arrest development at multi-cell aggregation.

## Major phenotyopic developmental defects in cells lacking *chdA*, *chdB* or *chdC*

To investigate CHD functions in *Dictyostelium*, we made deletion strains for each of the Chd genes; immunoblotting confirmed the absence of the targeted protein in each mutant strain (Fig. 1B). Mutants lacking each gene were viable, but possessed different developmental phenotypes that largely correlated with their peak expression patterns. Disruption of *chdA* causes a slight (~1 hour) delay in streaming maxima, but no apparent effect on aggregate, tip or slug formation during early *Dictyostelium* development. However, the *chdA* nulls exhibited a significant delay at the onset of terminal differentiation (i.e. culmination), ~16 hours post-starvation. This delay is best illustrated when *Dictyostelium* are developed on nitrocellulose filters where wild-type cells rarely develop migratory pseudoplasmodia, but instead rapidly progress to terminal differentiation. Under the same developmental conditions, *chdA*-null mutants form migratory pseudoplasmodia at high frequency (Fig. 1C) and persist at this stage for extended times, when compared with wild type.

ChdB is maximally expressed during early development and *chdB*-null mutants take ~2-4 hours longer to form multicellular mounds than do wild type. The timing defect does not appear to be stage specific; initiation of streaming, loose mound formation and tight aggregation are proportionally delayed. It is currently unclear how this phenotype originates. Beyond this developmental delay, no prominent phenotypic effects were apparent.

The strongest developmentally defective *chd*-null phenotype was observed with cells lacking *chdC*. *chdC*-null cells exhibited a delay during aggregation of ~2 hours and a developmental arrest at the multicellular mound stage, at ~12 hours; development failed to progress to the tipped mound stage, but instead formed bulbous extensions when development terminated (Fig. 1D). The developmental arrest in *chdC*-null mutant cells was completely penetrant, with no migratory pseudoplasmodia or mature terminal wild-type structures formed.

## Differential growth effects of the CHDs

During cell culture, we observed a slower growth rate for *chdC* nulls, than for the other strains in axenic suspension. To quantify

growth rates, we identically diluted wild type, and *chdA*, *chdB* and *chdC* nulls from log phase growth (~$2 \times 10^6$ cells/ml) into fresh growth media, and monitored growth over several days (Fig. 2A). The growth rate for *chdC* nulls was consistently halved compared with all other strains. The growth defect of *chdC* nulls is not due to deficiencies in cytokinesis, and *chdC* nulls are not multi-nucleate.

We considered whether *chdC* nulls might be aberrantly regulated during the transition from growth to development in response to nutrient depletion. Prior to the onset of starvation, *Dictyostelium* monitor the accumulation of various secreted factors to sense an increase in cell density (Burdine and Clarke, 1995). Inappropriate cell density sensing by *chdC* nulls could lead to growth defects or delayed entry into development and subsequent asynchrony. Using RNA and protein samples taken from growing wild-type cultures at ranges of ~$5 \times 10^4$ to $5 \times 10^6$ cells/ml, we show that the relative expression of both *ChdC* mRNA (Fig. 2B) and ChdC protein increases as cell density rises from ~$5 \times 10^5$ to $1 \times 10^6$ cells/ml. Thus, *ChdC* regulation appears linked to a pre-starvation response.

To determine whether ChdC function might additionally participate in the pre-starvation response, we examined cell density-dependent expression of the prototypic pre-starvation response marker Discoidin I (Burdine and Clarke, 1995) in wild-type and *chdC*-null cells. Discoidin I mRNA expression was similarly enhanced in wild-type and *chdC*-null cells at a cell density transition from ~$3 \times 10^5$ to $1 \times 10^6$ cells/ml (Fig. 2C). Although the data indicate that ChdC expression is induced by the pre-starvation response, as with several other early developmental markers, they do not support a requirement for ChdC to mediate this process.

## CHD effects on chemotaxis

To further assess CHD function during early development, we examined wild-type and *chd*-null cells that had been induced to differentiate in shaking culture in the presence of exogenous pulses of cAMP. These cAMP conditions mimic early stage developmental signaling and bypass the potential for defects in endogenous cAMP synthesis (McMains et al., 2008). Cells were then assayed for their ability to move by chemotaxis towards an exogenous cAMP source. Chemotactic behavior of *chdB* nulls is highly similar to wild type
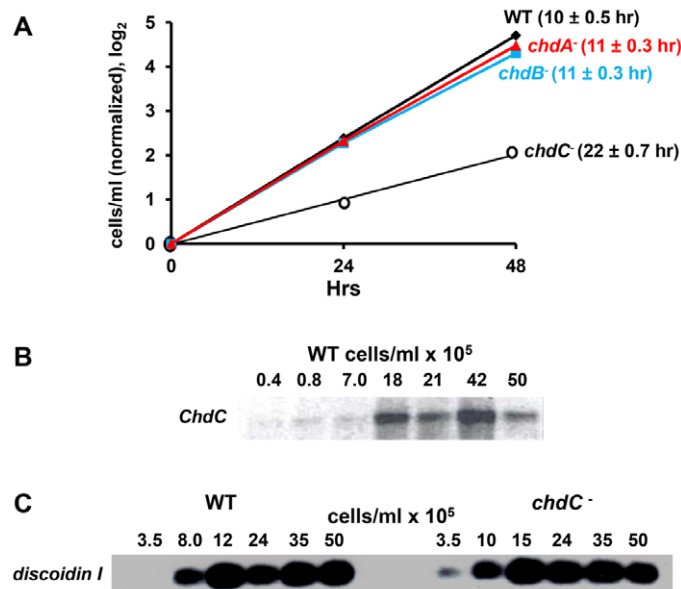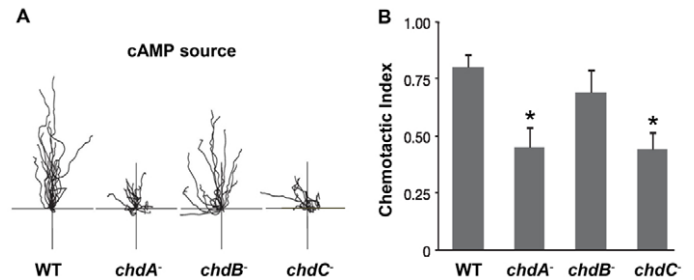
**Fig. 2. ChdC and cell growth.** (A) Wild-type, *chdA-*, *chdB-* and *chdC*-null cells were grown to log phase ($<2\times10^6$ cells/ml) and diluted ~20× into fresh media and cell aliquots taken at various times to evaluate relative growth rate. Growth increase for each strain was normalized to the initial cell density and plotted as a $log_2$ scale; doubling times for each strain are indicated. (B) RNA was prepared from growing wild-type cells at the indicated cell densities and hybridized by RNA gel blot with a *ChdC* probe. (C) RNA was prepared from growing wild-type and *chdC⁻* cells at the indicated cell densities and hybridized by RNA gel blot to a probe specific for the coding region of discoidin I.



**Fig. 3. Differential CHD functions during chemotaxis.** (A) Individual wild-type, *chdA*-null, *chdB*-null and *chdC*-null cells were imaged for chemotaxis toward a directed cAMP point source during a 15-minute period. Tracings reflect the migration paths for different cells of each strain. (B) The chemotactic index was plotted for each strain as mean±s.e.m. The Mann-Whitney test was used to evaluate significance. *$P<0.001$.

(Fig. 3). By contrast, both *chdA*- and *chdC*-null cells had significant chemotaxis defects, including reduced cell speed, increased cell turning and lower chemotactic indices. Nonetheless, chemotaxis to cAMP was not absent in either *chdA* or *chdC* nulls, consistent with their ability to form multicellular aggregates during development. The discrete phenotypes of each strain suggest that the individual CHDs have distinct actions, with only limited functional redundancy.

## Aberrant and unique transcriptional profiles of *chd* nulls during growth and early development

To examine directly differential effects on gene expression upon loss of ChdA, ChdB or ChdC, we applied high-throughput transcriptome sequencing (RNA-seq) for the three mutant strains, and compared relative mRNA transcript levels of each strain with that of wild type and with each other. Transcriptome profiles were determined for cells during growth and following 5 hours of stimulation with exogenous 100 nM pulses of cAMP. These latter conditions promote early differentiation and bypass the requirement for endogenous extracellular cAMP signaling (McMains et al., 2008).

During growth, ~10-15% of all genes are mis-expressed by over twofold ($P<0.05$) in each strain compared with wild type (Fig. 4A). Yet most wild-type gene expression is not altered upon loss of ChdA, ChdB or ChdC. When expression profile differences are compared between any two strains (Fig. 4A) or if all mis-expressed genes are collectively analyzed by comparative heat maps (Fig. 4B), they cluster as distinct gene sets. Although the gene sets for each strain are not fully unique, their observed intersections are no greater than would occur by the random distribution of independent variables. We conclude that ChdA, ChdB and ChdC predominantly

regulate distinct gene sets during growth. We did not detect compensatory expression profile changes of *chdA*, *chdB* or *chdC* mRNAs in the various mutant strains.

We next surveyed for functional gene classes that might be mis-expressed during growth of the various *chd* nulls, using Gene Ontology (GO) annotation. Despite the large number of genes that are downregulated in *chdA* and *chdB* nulls, little group biases could be discerned during growth. This is consistent with a lack of a significant growth phenotype for these strains. By contrast, *chdC* nulls displayed a poor growth phenotype and a large number of genes involved in mitochondrial and metabolic processes were poorly expressed exclusively in *chdC* nulls (Fig. 5A and supplementary material Fig. S3A).

Many regulators of aggregation also showed altered expression in growing *chdC* nulls. *ctnA*, *cf45-1* and *cf60*, three gene members of the secreted countin complex that potentiate inter-cell signaling and chemotactic movement (Brock and Gomer, 1999; Brock et al., 2003; Brock et al., 2006), were downregulated in *chdC* nulls. Expression of *lmcA*, *lmcB* and *srsA*, which function similarly to countin, was also suppressed, while expression of other modulators of aggregate size (e.g. *cnrJ*, *cnrG* and *cnrK*) was upregulated.

We also mapped gene expression patterns in *chdA*, *chdB* and *chdC* nulls that had been induced to synchronously differentiate by cAMP pulsing, to a time point akin to ~8 hours of development, when cells are chemotactically competent (Fig. 4C). As wild-type cells initiate development, there are large changes in the *Dictyostelium* transcriptome (Van Driessche et al., 2002; Loomis and Shaulsky, 2011). These changes are further augmented in the *chd* nulls, where individually 15-30% of all genes are mis-expressed by more than twofold ($P<0.05$). All of the mis-expressed genes were clustered in order to compare expression differences among all the cell lines, and displayed as heat maps (Fig. 4D). Although there is a trend for each strain to similarly exhibit more gene de-repression (i.e. overexpression) than repression (i.e. under-expression), the expression patterns for the *chdA*, *chdB* and *chdC* nulls display distinct gene expression profiles, underscoring their unique developmental phenotypes.

A complex series of interacting circuitries are shown to direct chemotactic movement and signal response to extracellular cAMP in *Dictyostelium* (Kortholt and van Haastert, 2008; McMains et al., 2008; Swaney et al., 2010). Although they primarily function downstream of the CAR1/Gα2βγ (cAMP receptor-G protein) complex, they diverge into several broad paths, including the cGMP-dependent and -independent actions of guanylyl cyclases
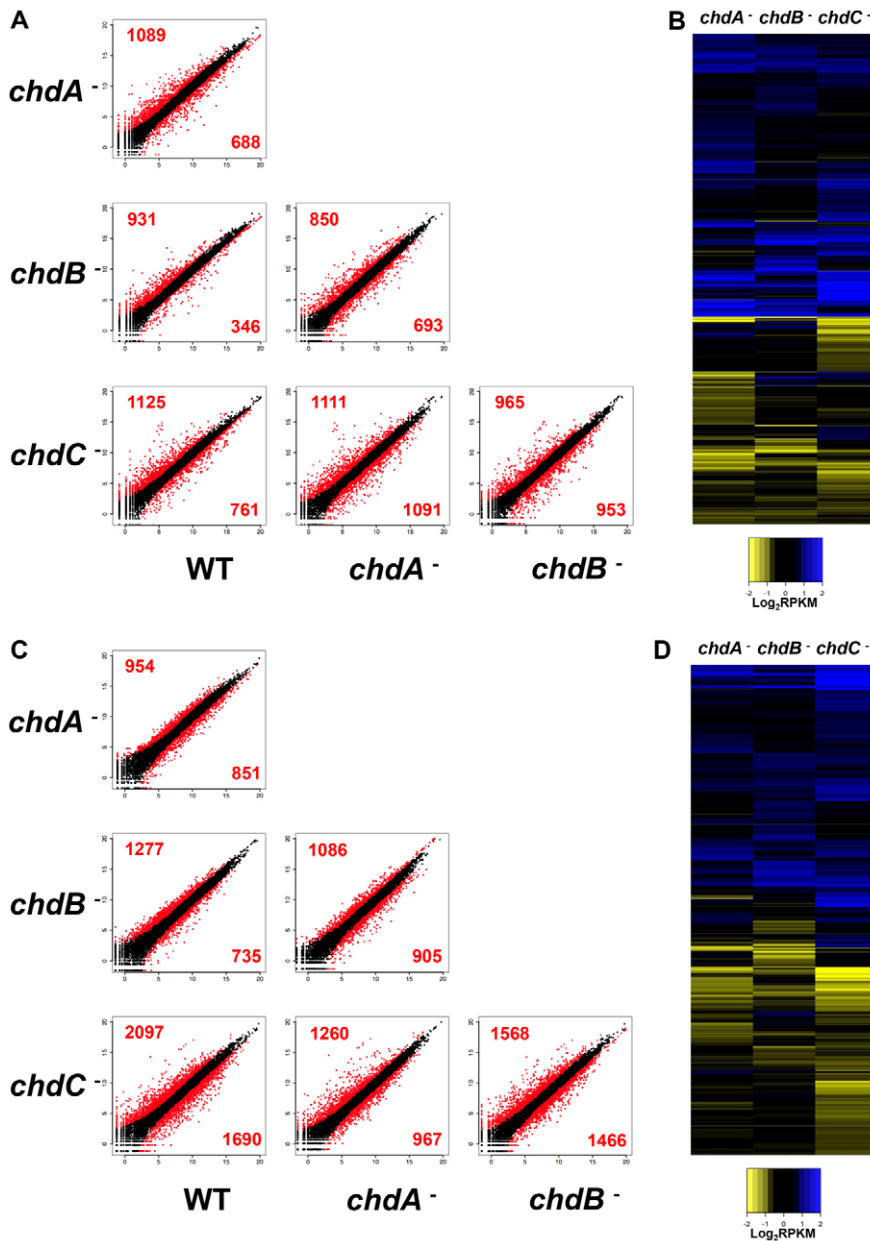
**Fig. 4. Large transcriptome differences among the *chd* nulls during growth or differentiation.** Scatter plots of normalized (RPKM, log$_2$) expression values for genes in wild-type, *chdA*-, *chdB*- and *chdC*-null cells during growth (A) or after 5 hours of cAMP-pulsing (C). Data points with more than twofold (*P*<0.05) expression differences between indicated strains are in red, and less than twofold (*P*<0.05) expression differences are black. Heat maps of log$_2$-fold changes in RPKM values in all genes with expression differences [≥2-fold (*P*<0.05)] in *chdA*, *chdB* or *chdC* relative to wild type, during growth (B) or after 5 hours of cAMP pulsing (D).

sGC and GCA, the PIP$_3$-dependent and -independent functions mediated via mTORC2, inter- and intracellular cAMP accumulation and signaling, and the interactions of Ca$^{+2}$ and phospholipase A$_2$ pathways (Kortholt and van Haastert, 2008). These networks have been defined biochemically and genetically and include ~50 genes. Of these, 50% are mis-expressed in *chdC* nulls (Fig. 5B), suggesting defects in chemotactic response, but also in extracellular cAMP signal relay (e.g. Gα2, PKBR1, CRAC, ERK2). Other gene classes that influence chemotaxis via cAMP wave propagation and cytoskeletal organization (supplementary material Fig. S3B,C) are also preferentially mis-expressed in *chdC* nulls, and to a lesser degree in *chdA* nulls. Thus, although the ChdC and ChdA gene sets are distinct, there are some shared elements. Yet as many more chemotactic genes are mis-expressed in *chdC*-nulls than in *chdA*-nulls, the data also explain the more minor streaming defects of *chdA* nulls during development. In addition, as several independent signaling pathways regulate chemotaxis during *Dictyostelium* development, their collective and compensatory activities can

dampen effects caused by deficiencies in selective elements (Van Haastert and Veltman, 2007; Kortholt and van Haastert, 2008). Accordingly, chemotaxis is compromised, but is not eliminated, in *chdA* and *chdC* nulls.

The culmination defect of *chdA* nulls is a post-slug formation, late developmental event. The regulatory pathways for this stage are complex (Harwood et al., 1992; Guo et al., 1999) and we do not observe gene expression defects at early stages that are predictive of the phenotype. Although *chdB* nulls show large numbers of mis-expressed genes, the cells do not exhibit a strong early phenotype and the mis-expressed genes do not group into simple GO collections.

cAMP signaling also directs developmentally regulated gene expression changes. Cells respond differentially to low (nM) level pulses of cAMP during early development and to non-varying, higher (μM) cAMP levels at the multicellular stage. Although only 13 genes are currently classified as exclusively pulse-dependent (Iranfar et al., 2003), 11 of these are under-expressed in *chdC*-nulls
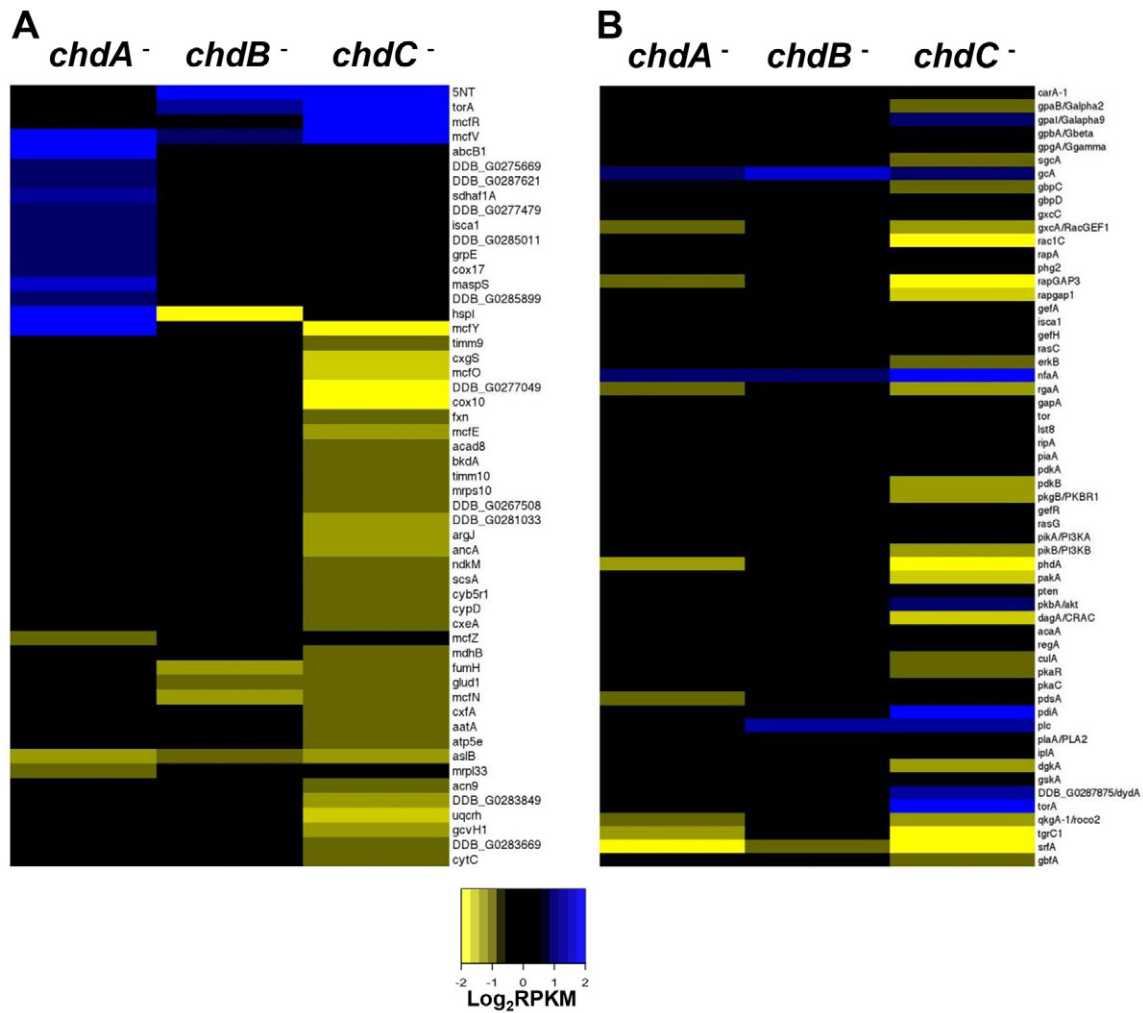
**Fig. 5. Expression changes in GO clustered genes for growth or development.** (A) Heat maps show log$_2$-fold changes in RPKM values for 53 genes with GO annotations associated with mitochondrial functions in the *chdA*, *chdB* and *chdC* nulls relative to wild type during growth. (B) Fifty-nine gene functions have been mapped to a cAMP chemotaxis network that acts downstream of CAR1 (carA). Heat maps show log$_2$-fold change in RPKM values for these genes in the *chdA*, *chdB* and *chdC* nulls relative to wild type, during differentiation in the presence of cAMP pulses for 5 hours. Genes are sorted approximately into separate signaling pathways downstream of the cAMP receptor.

and 10 are under-expressed *chdA* nulls (supplementary material Fig. S3C); only 1 gene is affected in *chdB* nulls. In contrast to pulse-regulated gene expression, aggregation and cell-type specific gene expression is mediated, in part, by response to a non-varying cAMP stimulus, involving regulatory genes such as *tgrC1/lagC*, *gbfA* and *srfA* (Fig. 5B). Only the *chdC* nulls show developmental arrest during aggregation and only *chdC* nulls show impaired expression of all these essential regulatory factors.

### ChdC-mediated regulation of multicellular development and pattern formation

The observed chemotaxis (see Fig. 3) and morphology (see Fig. 1) defects of the *chdC* nulls fit precisely with those identified during our initial genetic screens for aberrant chemotaxis and multicellular development. The RNA-seq data from the 5-hour pulsed set indicate poor expression of genes (e.g. *tgrC1/lagC*, *gbfA*, *srfA*) that are required for multi-cell differentiation. Indeed, the strongest phenotype of *chdC*-null mutant cells arises as cells begin multicellular development, where wild-type cells in the aggregation mound differentiate into the precursors of the terminally spore and

stalk cells. Prespore cells comprise ~80% of mound cells, with the remaining prestalk cells that give rise to the stalk, basal disc and supporting cup structures (Williams, 2006). We, thus, examined multi-cellular differentiation of the *chdC* nulls.

Endogenous prespore *pspA* and *cotB* and prestalk *ecmA* and *ecmB* mRNA (Williams, 2006) levels are diminished in *chdC* nulls compared with wild type (Fig. 6A). *In vivo* reporter expression using *pspA* promoter-*lacZ* fusions shows decreased reporter activity and restricted expression patterning compared with wild type (Fig. 6B). In addition, the expression of 53% of genes annotated as prespore-specific (including *pspA* and *cotB*) is halved in *chdC*-nulls as assayed by RNA-seq at the early mound stage, fully consistent with the defects described for prespore/spore differentiation (see Fig. 6).

Prestalk cell differentiation is more complex, marked by multiple sub-compartments of defined expression termed the pstA, pstO and pstB regions, which are primarily defined by subpromoter regions of prestalk genes *ecmA* and *ecmB* (Jermyn et al., 1987). In *chdC*-null mutants, *ecmA/lacZ* is expressed with a weaker and scattered pattern, without defined sorting to the tip of
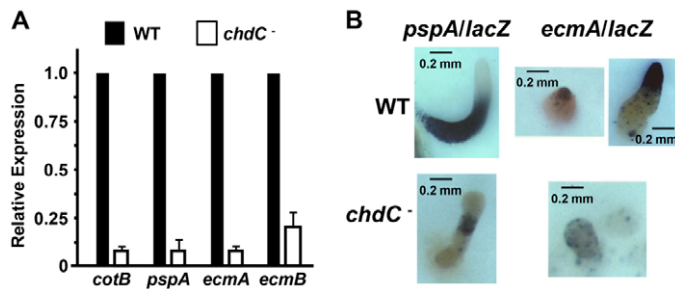
**Fig. 6. Regulation of prespore/spore and prestalk/stalk differentiation by ChdC.** (A) RNA was prepared from 12-hour developed cells, and relative expression levels of the prespore genes *pspA* and *cotB* and the prestalk genes *ecmA* and *ecmB* were quantified by RT-qPCR. (B) Wild-type and *chdC*-null cells were transformed with either the prespore-specific expression construct *pspA/lacZ* or the prestalk-specific expression construct *ecmA/lacZ*, plated for development on nitrocellulose filters and stained for β-galactosidase activity.

the mound (Fig. 6B). Global transcriptional profiling also gave a clear view of the observed broad prestalk defects, where the expression of 56% of genes annotated as prestalk specific is halved in *chdC* nulls.

Other GO terms that were enriched in the downregulated population of *chdC* nulls at the loose mound stage broadly segregate as regulators of cell division or metabolism. The former group includes cell cycle genes (*cdk1*, *cdc45*, *cycB*), DNA replication genes (*polA1*, *polA3*, *polD2*, *pcna*, *rcf3*, *rcf4*, *rcf5*) and mitotic genes (*nek2*, *kif2*, *kif12*). Although controversy surrounds the requirement of cell cycle progression during *Dictyostelium* development (Chen et al., 2004; Muramoto and Chubb, 2008), this gene set is significantly upregulated during wild-type development (Strasser et al., 2012).

## Autonomous and non-autonomous defects in *chdC*-nulls

The RNA-seq data indicate that *chdC* nulls have defects in both cell-cell signaling (e.g. cAMP production) and cell-autonomous (e.g. the TgrC1/LagC-GbfA) regulatory pathways. These data suggest that although certain developmental aspects of *chdC*-null development might be rescued by exogenous wild-type signals, they would be insufficient to bypass defects in cell-autonomous functions.

To examine possible defects in both cell-autonomous and non-autonomous pathways in *chdC* nulls, we investigated cell pattern specification in a series of chimeric, mixed cell developments. *chdC* nulls were mixed at various cell ratios with the parental wild type. Remarkably, although 100% *chdC* nulls are unable to form terminal fruiting body structures, chimeric organisms with as few as 2.5% wild-type cells will progress to fruiting bodies, albeit with a reduced spore mass and a significantly larger basal disk (Fig. 7A).

In chimeras with unmarked 90% *chdC* nulls, the 10% ubiquitously marked *rZIP/lacZ* (Balint-Kurti et al., 1997) wild-type cells preferentially, but not exclusively, populate the anterior prestalk region (Fig. 7B). In a reverse experiment, where the 90% *chdC* nulls are marked with *ecmA/lacZ* and mixed with 10% unmarked wild-type cells, stained *chdC* nulls can localize to the prestalk/stalk regions (Fig. 7C). These data suggest that the failure of tip formation in *chdC* nulls may result from defects in signal propagation and not simply from a loss of an autonomous intracellular function.

To investigate non-autonomous parameters further, we examined terminal cell formation under monolayer induction conditions (Kay, 1987). Stalk cell differentiation is induced by the sequential exposure of cells to first cAMP and then to DIF-1. Under these conditions ~55% of wild-type cells were induced to form vacuolated stalk cells in the presence of DIF-1, a process that was highly suppressed by simultaneous exposure to cAMP (Fig. 7D). By contrast, only ~10-12% of *chdC* nulls could form vacuolated cells in the presence of exogenous DIF-1, regardless of the presence of cAMP (Fig. 7D).
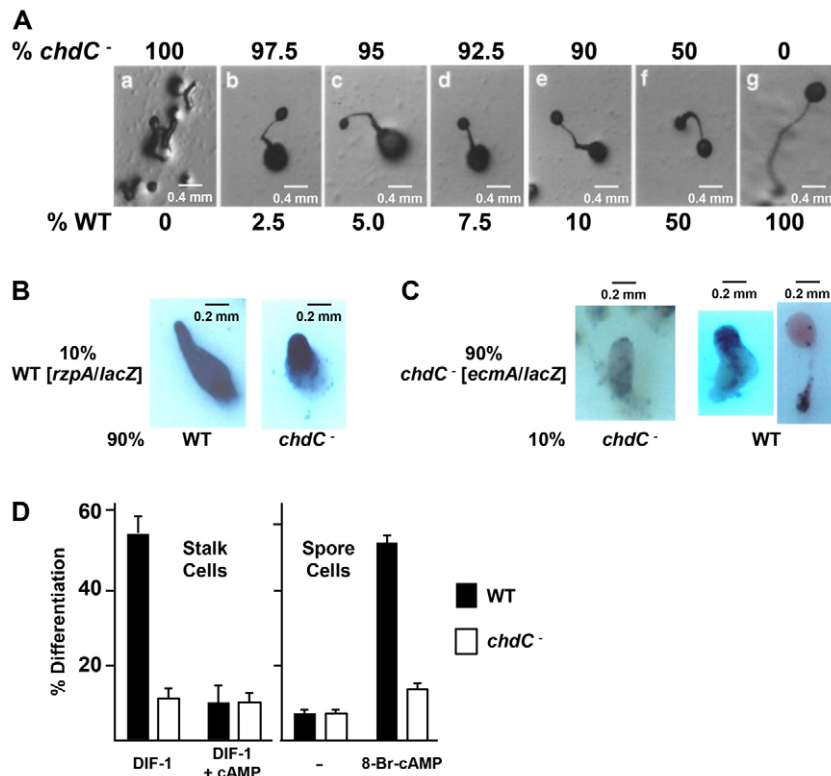


**Fig. 7. Developmental defects of *chdC* nulls are partially rescued by chimeric development with wild-type cells.** (A) The indicated percentages of wild-type and *chdC*⁻ cells were mixed prior to plating for development. Images were obtained after 36 hours of development. (B) Wild-type cells were transformed with the ubiquitously expressed *rzpA/lacZ* reporter construct, mixed and developed as a 10% ratio with 90% unmarked wild-type cells or with 90% unmarked *chdC*⁻ cells, and stained for β-galactosidase activity. (C) *chdC*⁻ cells were transformed with the prestalk-specific reporter construct *ecmA/lacZ*, mixed and developed as a 90% ratio with 10% unmarked wild-type cells or with 10% unmarked *chdC*⁻ cells, and stained for β-galactosidase activity. (D) Stalk cell assays: wild-type and *chdC*-null cells were plated at low cell density in monolayer with DIF-1 or with DIF-1 + cAMP. The percentage of cells that were fully vacuolated (mature stalk cells) was determined using phase-contrast microscopy. Spore cell assays: wild-type and *chdC*-null cells were plated at low cell density in monolayer without or with 8Br-cAMP to promote sporulation. Assays were scored by phase-contrast microscopy for the percentage of phase-bright fully mature spore cells.

In low cell density monolayers, spore formation can be directly induced with the membrane-permeant cAMP analog 8Br-cAMP, which directly activates cAMP receptors and the cAMP-dependent protein kinase (PKA) and bypasses other signaling requirements (Kay, 1987). Under these conditions ~60% of wild-type cells differentiated into spores, whereas only 15% of *chdC* null cells formed spores, efficiencies similar to that of uninduced wild-type cells (Fig. 7D), indicating an intrinsic loss in spore-forming ability of *chdC* nulls.

We also examined spore differentiation following chimeric development of 10% wild type with 90% *chdC* nulls. Spores were harvested and germinated in the absence and presence of blasticidin to differentiate and quantify wild-type and *chdC* nulls. Here, fewer than 10% of total spores from the mix were blasticidin-resistant *chdC* nulls. Although spore differentiation is not totally blocked in *chdC* nulls, it was substantially reduced to a frequency of ~10% that of WT, confirming a cell-autonomous defect for spore differentiation in *chdC* nulls. Although exogenous wild-type cells are able to influence developmental patterning in *chdC* nulls, they are unable to rescue terminal cell differentiation of *chdC* nulls.

## DISCUSSION

Of the multiple SWI2/SNF2, ATPase-type chromatin remodeling proteins in eukaryotes, the CHD family is among the largest and most diverse (Ryan and Owen-Hughes, 2011). Humans have nine distinct CHDs that group into three structural subfamilies, CHD I, CHD II and CHD III. The CHD I subfamily is ubiquitous, found in the yeasts, plants and metazoa, whereas CHD II and CHD III members are generally restricted to multicellular organisms, with the latter seemingly absent in plants (Ryan and Owen-Hughes, 2011). It is, thus, striking that *Dictyostelium*, a facultative metazoan, encodes two proteins that cluster with the CHD III subfamily. Accordingly, *Dictyostelium* provides a unique model system for analyzing multiple and diverse regulatory aspects of different CHD family members; *Dictyostelium* does not seem to encode CHD II members, which are defined by the presence of PHD fingers (Ryan and Owen-Hughes, 2011).

Although CHD I members are suggested to facilitate an open chromatin organization at active promoters and to regulate nucleosome spacing more distally (Hennig et al., 2012; Persson and Ekwall, 2010; Zentner et al., 2013), their interactions at these promoter sites do not seem to be functionally essential at a global level. *S. cerevisiae* are viable without CHD1 (Tsukiyama et al., 1999) and *D. melanogaster* that lack CHD1 develop to maturity, although both males and females are sterile (McDaniel et al., 2008). Nonetheless, mouse ES cells depleted of CHD1 exhibit defects in pluripotency (Gaspar-Maia et al., 2009). Although *Chd1*$^{-/-}$ mice have not been reported, *Chd2*$^{-/-}$ mice are embryonically lethal (Marfella et al., 2006b). Therefore, CHD I family members can have crucial functions in the control of developmentally essential gene sets.

*chdA*-null *Dictyostelium* are viable, without apparent impact upon growth on bacteria or in axenic culture. However, *chdA* nulls exhibit a highly defined early developmental defect, involving poor transcriptional response to the extracellular activating signal cAMP. Furthermore, the morphological transitions and cell fate determinations required for terminal differentiation, which occur co-temporaneously with maximal levels of *ChdA* expression in wild-type cells, are effectively arrested in cells lacking ChdA. To some extent, the phenotypic effects of ChdA (i.e. a CHD1/2 ortholog) loss in *Dictyostelium* parallels the essential developmental requirement for CHD I members in mouse.

Consistent with the observed phenotypes, we also show that loss of *ChdA* in *Dictyostelium* causes a restricted, rather than global, impact on gene expression. Fewer than 15% of the *Dictyostelium* genome shows altered patterns of gene expression during growth or early development when parental and *chdA*-null cells are compared. We observe a similar distribution of genes that exhibit up- or downregulation. The data indicate that ChdA in *Dictyostelium*, and potentially CHD1 in other regulatory networks, is not essential for global chromatin organization and gene expression induction. ChdA is likely to have more confined operational targets and integrate with other chromatin organizing components.

Studies of the CHD III class in *Drosophila* (e.g. Kismet) or in humans (e.g. CHD7) suggest unique developmental functions compared with other CHD members. Kismet regulates cell fate determination of *Drosophila* and can antagonize the repressive effects of Polycomb in homeotic patterning (Srinivasan et al., 2008). Similarly, loss-of-function mutations or haploinsufficiencies of *CHD7* in mouse, humans and zebrafish (Layman et al., 2010; Patten et al., 2012) have complex developmental defects. In humans, the eye, heart, ear and genitalia are specifically affected, and many phenotypes are recapitulated in mouse and zebrafish. Although CHD7 is not essential for self renewal, pluripotency or somatic reprogramming of mouse ES cells, it has been suggested that a significant defect in human CHARGE syndrome may involve lineage effects in the proliferation, cell migration and specification of neural crest cells (Zentner et al., 2010).

As in the metazoa, *Dictyostelium* has multiple (ChdB and ChdC) CHD III subfamily members, with ChdC having the most essential and complex regulatory functions of all CHD members. At the phenotypic level, *chdC*-null cells are viable, but have reduced growth rates. They respond to nutrient depletion for induction of multi-cell development, but rapidly display abnormalities that temporally parallel the upregulation of ChdC in wild-type cells. Response to cAMP, chemotaxis and aggregate formation are impaired, as are cell-fate commitments.

Many of the defects in *chdC* nulls can be directly attributed to specific abnormalities in gene expression patterns compared with parentals. Although only a small population of genes are mis-regulated during growth of *chdC* nulls, they include a large number of cellular metabolic and energetic regulators. By contrast, the mis-regulated genes in *chdB* nulls do not simply cluster in a few functionally related groups, but are distributed broadly among GO classes, and, under the conditions studied, ChdB seems to be only minimally required.

During aggregation, *Dictyostelium* organize a complex inter-cell signaling pathway, which directs chemotaxis and multi-cell aggregation, but also cytodifferentiation and morphogenetics. Central to this pathway is the production and secretion (signal-relay) of the chemoattractant cAMP (Kortholt and van Haastert, 2008; McMains et al., 2008; Swaney et al., 2010). Receptor-mediated response to cAMP involves a highly integrated pathway that collectively promotes signal amplification, cytoskeletal reprogramming, cell polarization and non-terminal, cell-fate specification; many individual components of the cAMP signaling pathway are required for all cellular responses (Kortholt and van Haastert, 2008; McMains et al., 2008; Swaney et al., 2010). We show that ~50% of the genes, which lie most immediate to receptor sensing, are mis-regulated in *chdC* nulls. Their mis-regulations are predicted to compromise cell-autonomous requirements of intracellular signaling that directs chemotaxis and differentiation, but also the non-autonomous, extracellular elements involved in cAMP signal relay. Thus, although certain non-autonomous defects of *chdC*

nulls may be rescued by co-development with a small population of wild-type cells, the cell-autonomous pathways that are regulated by intracellular signaling remain compromised even in the presence of exogenous cAMP or wild-type factors.

Gene expression patterns have also been studied in $Chd7^{+/+}$ and $Chd7^{-/-}$ mouse ES cells (Schnetz et al., 2010). Data indicate an impact on only a restricted gene set, with significant enrichment of genes primarily expressed in the ES population (Schnetz et al., 2010). In addition, more CHD7-binding sites map to enhancers of these genes than to non-ES class genes. Interestingly, these ES-enriched and CHD7-bound gene classes are generally upregulated in the absence of CHD7, suggesting a modulating repressive function on this ES gene set. Nonetheless, CHD7 is not entirely repressive, as other gene sets are downregulated upon the loss of CHD7. Regardless of the impact of CHD7 on ES cell transcriptional patterns as both a positive and negative effector, processes of self renewal, pluripotency and somatic cell reprogramming are not impaired in $CHD7$-deficient ES cells (Schnetz et al., 2010).

The directed and interactive roles of the different CHDs in multicellular differentiation are complex and still largely undefined. *Dictyostelium* provides a unique system to separately dissect these functions. We have shown that phenotypes resulting from deficiencies of ChdA, ChdB or ChdC in *Dictyostelium* are not global and, thus, parallel those in the more complex metazoa. It is particularly striking that proliferative, migration and cell specification defects of *chdC*-null *Dictyostelium* are mirrored in $Chd7^{-/-}$ neural crest cells. We further speculate that defective inter- and intra-cell signaling, as seen in *chdC*-null *Dictyostelium*, may also contribute to the developmental abnormalities seen in CHARGE syndrome.

Our data extend conclusions on the role for CHDs in gene expression. Each of the CHDs in *Dictyostelium* regulates different developmental patterns and different gene sets. The CHDs do not appear to act globally and can have both repressive and activating functions. We suggest that the mechanistic functions of CHD in *Dictyostelium* are similarly shared in the metazoa and that more-complete analyses will contribute significantly to the understanding for the ultimate treatment of genetic diseases, such as CHARGE syndrome, caused by loss-of-function mutations in *CHD* loci.

## MATERIALS AND METHODS

### Bioinformatics of CHD and SWI2/SNF2-related proteins

Identification of genes in *Dictyostelium* was performed by BLAST search of the *Dictyostelium* genome (http://dictybase.org/tools/blast). Three CHD proteins were identified: ChdA is DDB_G0284171, ChdB is DDB_G0280705 and ChdC is DDB_G0293012 (Basu et al., 2013). Additional domains were identified using EMBL SMART software (Letunic et al., 2012) and NCBI conserved domain software (Marchler-Bauer et al., 2011). The phylogenic tree was created using http://phylogeny.lirmm.fr/phylo_cgi/index.cgi (Dereeper et al., 2008) and full-length proteins. Similar clusterings were generated using only the chromodomains or helicase motifs.

### Dictyostelium strains

*Dictyostelium* Ax2 (wild-type) cells were grown axenically in HL5 medium at 20°C. Gene deletion constructs were produced using standard PCR-based methods. Gene regions were disrupted by the floxed blasticidin resistance cassette from pLPBLP (Faix et al., 2004) using TOPO-Blunt II (Invitrogen). Gene deletion strains were generated by transformation with 15 μg of linearized plasmid using electroporation and selection in HL5 media supplemented with 10 μg/ml Blasticidin S. Transformants were screened by PCR and gene disruptions confirmed by immunoblotting.

### Dictyostelium development and phenotype analyses

To analyze development, growing cells in log phase ($1$-$3 \times 10^6$ cells/ml) were washed twice in KK2 buffer (15 mM $KH_2PO_4$, 3 mM $K_2HPO_4$) and developed on 0.45 μm nitrocellulose filters. Developmental expression of the individual CHD proteins were analyzed every 2 hours by immunoblotting. Slug migration assays were carried out in a 'slug can'. Washed cells were plated at one edge of a nitrocellulose filter, developed to the slug stage, and subjected to unidirectional light; images were taken after 48 hours. For chemotaxis, $5 \times 10^7$ cells/ml were shaken in KK2 buffer for 5 hours while being pulsed every 6 minutes with cAMP to a final concentration of $10^{-7}$ M. The cells were then placed in the Zigmond chamber (Z02; Neuro Probe, Gaithersburg, MD) in a cAMP gradient (source at $10^{-6}$ M cAMP, sink with no cAMP). Differential interference contrast (DIC) images of cells were captured at 6-second intervals for 15 minutes. Cell movement was analyzed using Dynamic Image Analysis System (DIAS) software (version 3.4.1) (Soll Technologies, Iowa City, IA; (Wessels et al., 2009). Stalk and spore cell differentiation in monoloayer assays were performed at a cell density of $1 \times 10^4$ cells/cm² (Kay, 1987) and scored by phase-contrast microscopy.

### Antibodies and immunoblotting

Antibodies specific to ChdA, ChdB and ChdC were raised in rabbits by immunizing with peptides (~20 amino acids) specific for each protein (Openbiosystems, 70 day protocol). Immunoblotting was by standard methodologies, using whole-cell, SDS lysates, 3-8% Tris-acetate gels and blotting overnight at low voltage.

### RNA-seq; sequencing and bioinformatics

RNA was isolated from all strains using TRIzol (Life Technologies). RNA quality was analyzed using the Agilent 2100 Bioanlyzer. 5 μg of total RNA was used for the Illumina libraries. RNA was polyA enriched and libraries were single-end, sequenced for 50 bases, using the Illumina HiSeq2000. Filtered reads from the manufacturers recommended pipeline (version 1.7) were aligned to the *Dictyostelium* genome with Tophat (version 1.3.0) (Trapnell et al., 2009) while masking the duplicated region of chromosome 2 (nucleotides 3,015,984 to 3,768,555) using the gene models from dictybase (http://dictybase.org/) (Basu et al., 2013). Reads were counted and normalized, and differential expression was calculated with HTSeq and DESeq (Anders and Huber, 2010). Heat maps were generated with the heatmap.2 function in the gplots package (Warnes, 2012) scatterplots were also produced in R (R Development Core Team, 2012). GO term enrichment used Orange (Curk et al., 2005). The RNA-seq data have been deposited in the GEO database (Accession Number, GSE47222).

### Author contributions
J.L.P., B.J.R., K.C.R., A.J.H. and A.R.K. developed the concepts and approaches. J.L.P., B.J.R., K.C.R. and A.R.K. performed experiments. J.L.P., B.J.R., K.C.R., A.J.H. and A.R.K. analyzed the data. J.L.P., A.J.H. and A.R.K. prepared the manuscript prior to submission.

### Supplementary material
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