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The P450 oxidoreductase, *RedA*, controls development beyond the mound stage in *Dictyostelium discoideum*

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Abstract

Background: NADPH-cytochrome-P450 oxidoreductase (CPR) is a ubiquitous enzyme that belongs to a family of diflavin oxidoreductases and is required for activity of the microsomal cytochrome-P450 monooxygenase system. CPR gene-disruption experiments have demonstrated that absence of this enzyme causes developmental defects both in mouse and insect.

Results: Annotation of the sequenced genome of *D. discoideum* revealed the presence of three genes (*redA*, *redB* and *redC*) that encode putative members of the diflavin oxidoreductase protein family. *redA* transcripts are present during growth and early development but then decline, reaching undetectable levels after the mound stage. *redB* transcripts are present in the same levels during growth and development while *redC* expression was detected only in vegetative growing cells. We isolated a mutant strain of *Dictyostelium discoideum* following restriction enzyme-mediated integration (REMI) mutagenesis in which *redA* was disrupted. This mutant develops only to the mound stage and accumulates a bright yellow pigment. The mound-arrest phenotype is cell-autonomous suggesting that the defect occurs within the cells rather than in intercellular signaling.

Conclusion: The developmental arrest due to disruption of *redA* implicates CPR in the metabolism of compounds that control cell differentiation.

Background

NADPH-cytochrome-P450 oxidoreductase (CPR, EC 1.6.2.4) is a ubiquitous enzyme that is required for activity of the microsomal cytochrome-P450 (CYP) monooxygenase system [1,2]. This system is involved in the metabolic activation and/or detoxification of numerous foreign compounds as well as in the metabolism of endogenous substrates, such as steroids, alkaloids and fatty acids [3,4]. CPR belongs to a family of diflavin oxi-

doreductases which also includes the flavoprotein subunit of bacterial sulfite reductase (SiR) as well as a methionine synthase reductase and the cytoplasmic NADPH-dependent diflavin oxidoreductase 1 (NDOR1) identified in eukaryotic cells [5-8]. In addition, the diflavin reductase domain is found in fusion with cytochromes P-450 or with hemoprotein forming complex multidomain enzymes such as the cytochromes P450BM3 and the nitric oxide synthases [6].

CPR is a membrane anchored ~78 kDa enzyme which contains one molecule each of FAD and FMN bound as prosthetic groups that facilitate transfer of electrons of NADPH to the prosthetic heme group of CYP [1,2,9]. CPR is also involved in transferring electrons to other molecules, including heme oxygenase, squalene epoxidase and cytochrome b₅ [10-12].

Despite the diversity of CYP isoforms that can be found in a single species [13], CPR in most organisms, except in certain plants and some zygomycetes, is encoded by only one gene [14-23]. Inactivation of the single-copy CPR gene in *Saccharomyces cerevisiae* results in mutants that accumulate only 25% as much ergosterol as observed in wild-type strains which probably accounts for the increased sensitivity of these mutants to the antifungal drug ketoconazole [16,24]. Moreover, it has been reported that cytochrome b₅ gene can suppress the phenotype resulting from disruption of the CPR gene and therefore might function as an alternative electron donor for CYP activity in yeast [25-27]. In the fungus *Gibberella fujikuroi* loss of CPR leads to a reduced growth rate and has a strong influence on gibberellin biosynthesis [20].

CPR gene-disruption experiments in mouse have demonstrated that absence of this enzyme causes defects leading to mid-gestational lethality [28-30]. *In situ* hybridization studies have shown high levels of CPR expression in mesenchymal cells of the limbs and developing olfactory neuroepithelia [31]. CPR has been implicated in odorant clearance in insect antennae [17] and in ecdysone 20-hydroxylation during insect embryonic development [32].

Annotation of the sequenced genome of *D. discoideum* [33] revealed the presence of three genes that encode putative members of the diflavin oxidoreductase protein family. DDB0187719 (*redC*) on chromosome 5 encodes a polypeptide of 633 amino acids, which is 56% similar to human NADPH-dependent diflavin oxidoreductase 1 (NDOR1), a cytoplasmic enzyme highly expressed in cancer cell lines with as yet unknown functions [8]. Two genes, *redA* (DDB0215407) on chromosome 6 and *redB* (DDB0190667) on chromosome 1, show about 50% similarity to CPR proteins in humans, rats, *Drosophila* and yeast. We found that inactivation of *redA* in *Dictyostelium* results in developmental arrest at the mound stage.

Results and Discussion

Identification of the disrupted gene in the REMI mutant *redA*⁻

The mutant described in this work was isolated from a REMI-mutagenic library screen for morphological mutants of *Dictyostelium discoideum*. Strain DG1047 was picked because it forms yellow mounds that fail to make

proper fruiting bodies. A portion of the disrupted gene was isolated from this strain by plasmid rescue in *E. coli* [34]. This fragment was used to screen a cDNA library prepared from vegetative cells. The largest cDNA insert (2094 bp) was sequenced and found to encode a putative protein of 631 amino acids with ~50% similarity to CPRs from human, rat, *Drosophila*, and yeast (Figure 1). The gene was designated *redA* as a mnemonic that it is likely to act in a redox reaction.

The *D. discoideum* CPR encoded by *redA* shows considerable conservation in the regions proposed to be involved in binding FMN, FAD and NADPH [2,9,18,35-37]. It is worth mentioning that the NADPH-2 region pointed out in Figure 1 contains the three residues Ser-596, Arg-597 and Lys-602 (positions numbered according human CPR) involved in the binding of the enzyme to the 2' phosphate of NADPH [2,38]. In addition, the conserved carboxi terminal motif G/K/N-R-Y-x-x-D-V/T-W is present in *D. discoideum* CPR. It has been demonstrated that the tryptophan in this motif plays a major role in discrimination of NADPH [36].

Analysis of the predicted RedA amino acid sequence by Signal P and TMHMM programs [39,40] revealed a N-terminal hydrophobic segment of ~20–25 amino acids (Figure 1) that should be sufficient for its anchorage to a membrane. Despite the fact that the hydrophobic N-terminal of mammalian CPRs is approximately 56 amino acids long [2], in plants and in fungi a shorter hydrophobic N-terminal is sufficient for membrane interaction [24,41-43]. Moreover, it has been proposed that interaction of human CPR to membranes and to CYPs is likely to involve additional hydrophobic patches on CPR surface [2,44].

Southern blot analysis of *D. discoideum* genomic DNA cleaved with a variety of restriction enzymes showed that RedA is encoded by a single copy gene (data not shown). Comparison of *redA* cDNA sequence with the *Dictyostelium* genome sequence [33] confirmed this result and showed that *redA* is an intronless gene located on chromosome 6. In most organisms analyzed, such as humans, mouse, *Drosophila*, *S. cerevisiae* and filamentous fungi, the CPR gene is present as a single copy and in the fruit fly two alternative splicing isoforms have been identified [14-18,20,45]. On the other hand, plants and certain fungi often have multiple copies of CPR gene [21,22,41,43,46,47]. We found two CPR genes in the *Dictyostelium* genome, *redA* and *redB*. Even though the amino acid sequences of *redA* and *redB* are 52% similar to each other, their nucleotide sequences are highly diverged and the genes appear to have evolved independently for a long time. *D. discoideum* genome has a third gene (*redC*) that encodes an additional member of diflavin oxidoreduct-

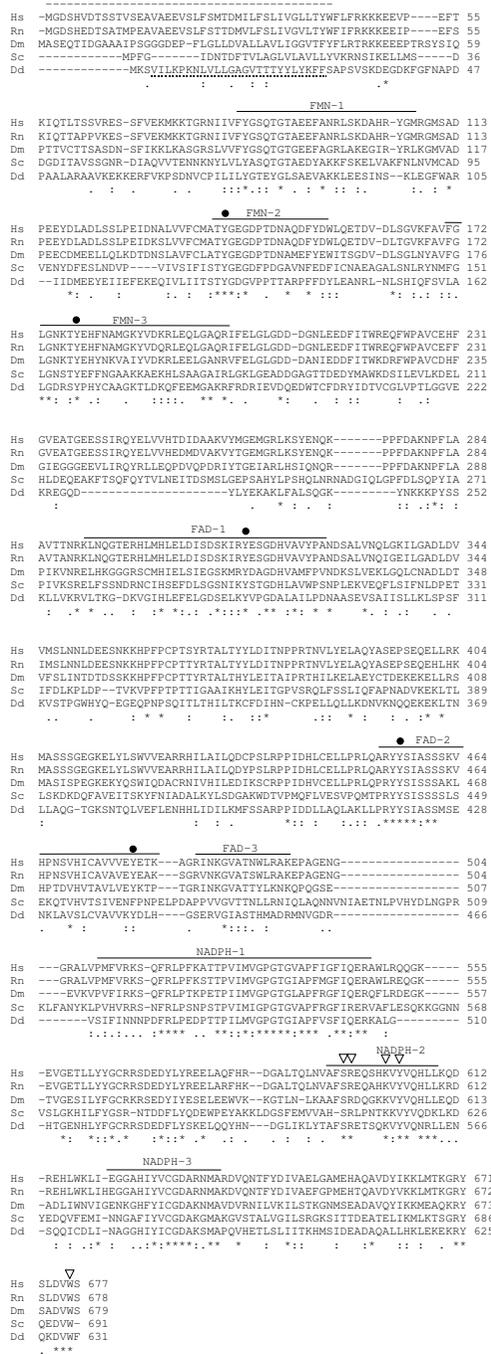


Figure 1
Alignment of CPR amino acid sequences. Comparison of *Dictyostelium discoideum* (Dd) NADPH cytochrome P450 oxidoreductase (RedA) amino acid sequence with orthologs from *Homo sapiens* (Hs, P16435), *Rattus norvegicus* (Rn, P00388), *Drosophila melanogaster* (Dm, CAA63639) and *Saccharomyces cerevisiae* (Sc, P16603) using CLUSTAL W program. Identical and conserved amino acids are denoted with (*) and (:), respectively. Semi-conservative changes are indicated with a single dot. Binding domains for FMN, FAD and NADPH are indicated. Closed circles indicate highly conserved aromatic amino-acid residues that are particularly important in flavin binding. Open triangle points residues involved in NADPH discrimination. The ~56 amino acid and the ~20 amino acid hydrophobic segments at the N-terminal are indicated by dashed line in human CPR and dotted line in *Dictyostelium* CPR, respectively.

ases family which conserves sequences defined as binding domains for FMN, FAD and NADPH but lacks the N-terminal hydrophobic region found in RedA and RedB.

Expression of *redA* during growth and development

The expression of *redA* was monitored by Northern blot using a *redA* cDNA fragment as probe. As shown in Figure 2, a single mRNA species of 2.3 kbp was present in growing cells and decreased in abundance upon starvation of the cells on filter pads. No *redA* mRNA could be detected late in development (Figure 2 and 3A) in agreement with the *redA* expression profile determined on microarrays [48]. As a control, we probed for the *csaA* mRNA encoding the cell adhesion protein gp80 which is highly expressed during early aggregation [49]. This mRNA accumulated rapidly to reach peak levels by 2 hours and decreased after 4 hours of development (Figure 2).

In contrast, we found that *redB* is constitutively expressed throughout development of AX4 cells, while *redC* is expressed at detectable levels only in vegetative growing cells being repressed upon cell starvation. As a late development marker we monitored expression of *ecmA* [50], a prestalk-specific gene (Figure 3A).

CPR mRNA has distinct expression patterns during the development of several tissues and organs in mice, and this expression is not coordinated with expression of CYP genes [29,31,51,52]. The CPR gene is expressed in the early stages of embryonic development, suggesting that CPR-dependent processes may be important at this stage of the embryogenesis [28,31,32,53]. In *Drosophila mela-*

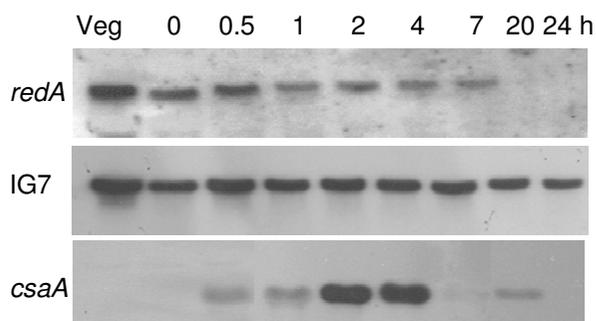


Figure 2
Expression of *redA* during growth and development of wild type AX4 cells. Exponentially growing AX4 cells (Veg) were starved on filter pads and harvested at the indicated times (h) after starvation. Identical Northern blots of total RNA samples were probed with *redA*, IG7 and *csaA* cDNAs as indicated. IG7 transcript is expressed at similar levels throughout the *D. discoideum* development [59].

nogaster the CPR gene shows high levels of expression in various embryonic tissues as well as in the antenna of adults [17]. In parsley and *Arabidopsis* there are two CPR genes, one of which is constitutively expressed while the other is induced by biotic and abiotic stresses [21,47].

Phenotype of the *redA* minus mutants

To confirm that the *redA*⁻ phenotype is due to the disruption of *redA*, we generated new mutant strains by homologous recombination with the original plasmid pRED isolated from *redA* REMI mutant. Effective *redA* disruption was checked by Southern blot analyses of genomic DNA from blasticidin-resistant clones (data not shown) and six independent knockout clones were isolated which showed the same mound-arrest phenotype. One strain (*redA*-KO) was selected for further analyses. As shown in Figure 4A, when compared to wild type AX4 strain, the majority of cells of *redA*⁻ and *redA*-KO mutants failed to make mature fruiting bodies after 24 h development on filters, and was arrested at the mound stage where they accumulated a yellow pigment (Figure 4B). It should be pointed out that the *redA*-KO mutants form a few tipped aggregates in the mound population after 48 hours starvation (data not shown). On the other hand, the original REMI and the recapitulated mutants did not show any significant differences in their growth curves when compared to the wild type AX4 (data not shown).

As expected, *redA*⁻ and *redA*-KO mutants did not express *redA* mRNA (Figure 5A). Despite their developmental defect, *redA*⁻ cells expressed *csaA* during development on filter pads (Figure 5B). Moreover, *redC* transcriptional profile is reasonably similar in wild type AX4 and in *redA*⁻ cells (Figure 3) as its transcript levels strongly decrease upon starvation. On the other hand despite being expressed throughout development both in AX4 and *redA*⁻ cells, *redB* transcript accumulates at higher levels in the latter (Figure 3). Also the peak of expression of *ecmA* was found to be advanced by four hours in the *redA*⁻ strain as compared to the wild type (Figure 3).

The developmental defect of *redA*⁻ mutant is not rescued by mixing with AX4 wild type cells. As shown in Figure 6, mixing 10% or 20% of AX4 with *redA*⁻ mutant did not overcome the mutant mound arrest indicating autonomy of the mutant phenotype. Moreover, the mutant cells did not inhibit wild type cells from forming fruiting bodies when they were developed together in equal numbers (not shown).

As mentioned above both *redA*⁻ and *redA*-KO mutants form yellow mounds upon starvation (Figure 4B). We have observed that after 48 hours filter starvation the mounds and even the filter turn a strong yellow colour. This does not reflect premature spore formation since the

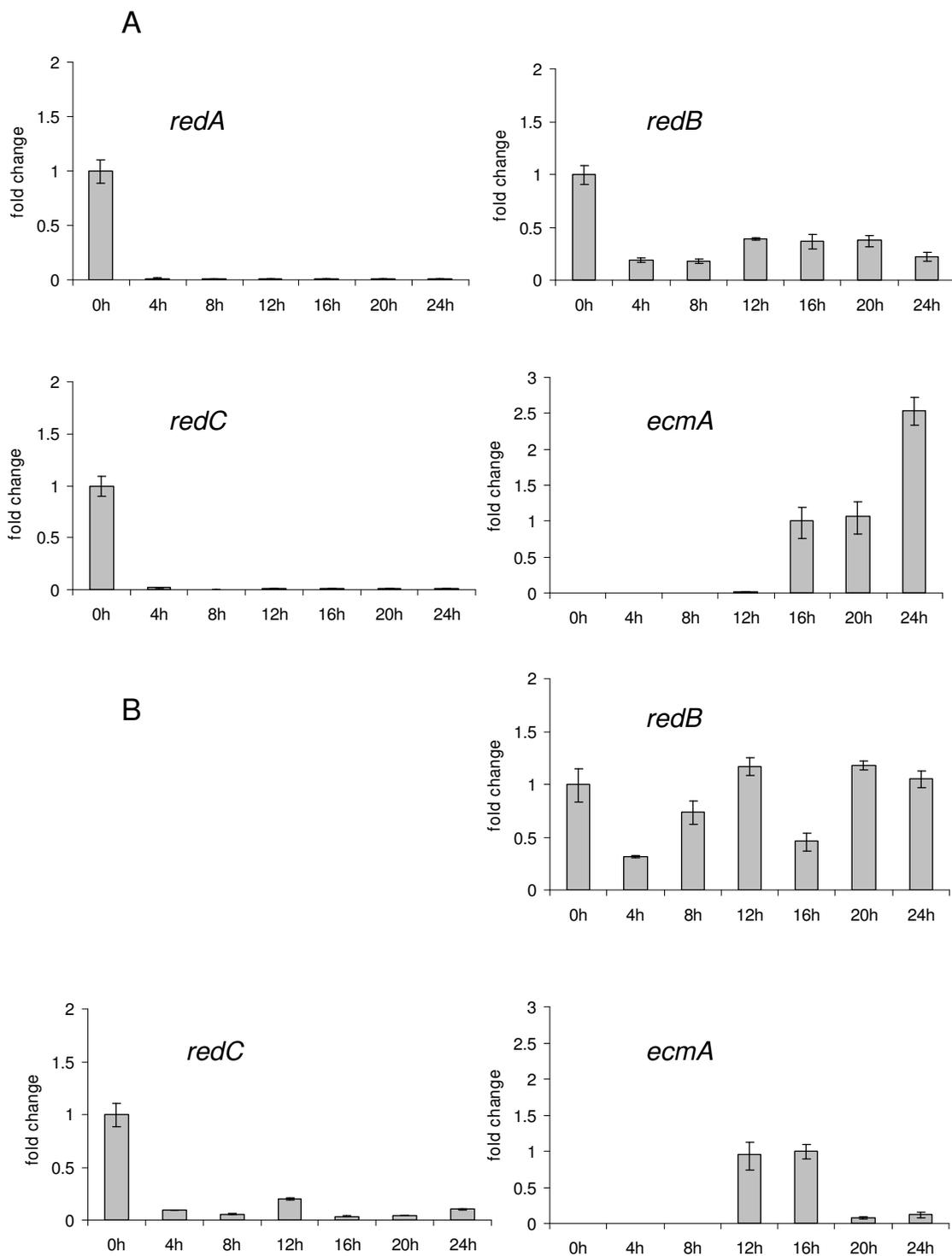


Figure 3
Transcriptional profile of *redA*, *redB*, *redC* and *ecmA*. Exponentially growing AX4 (**A**) and *redA*⁻ (**B**) cells were starved on filter pads and harvested at the indicated times (h) after starvation. Transcript levels for *redA*, *redB* and *redC* genes are relative to 0 h cells. Fold change for *ecmA* are relative to transcript levels detected at 16 h. Error bars represent the standard deviation from two independent experiments where qPCR assays were performed in triplicate.

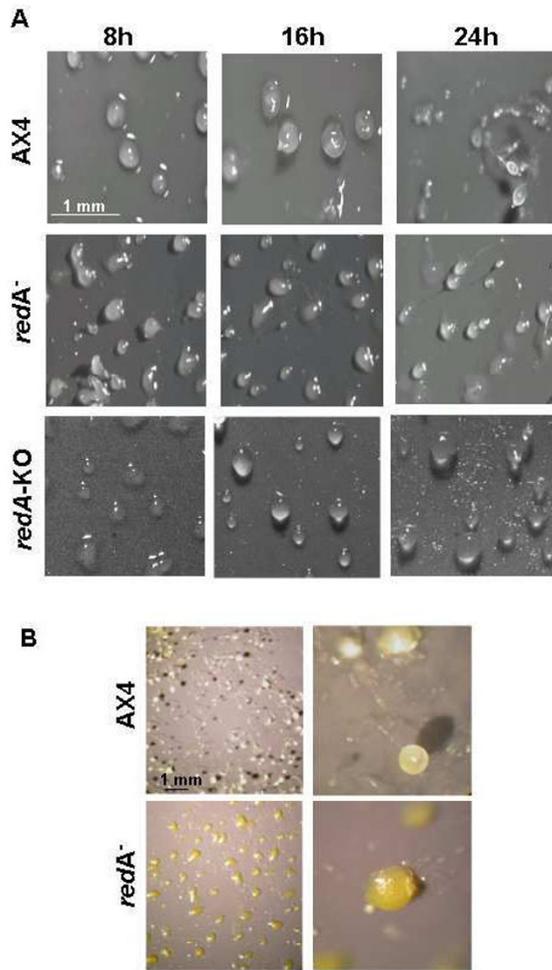


Figure 4
Disruption of *redA* impairs development at mound stage. (A) Exponentially growing AX4 wild type cells and the mutants *redA*⁻ and *redA*-KO were starved on filter pads and photographed at the indicated times (h) after starvation. (B) AX4 fruiting bodies and *redA*⁻ yellow mounds after 48 hours starvation on filter pads are shown at lower (left) and at 5× higher magnification (right).

yellow mounds do not contain any viable spores (data not shown). Chloroform extracts of *redA*⁻ mounds collected after 48 hours starvation show an absorption peak at 400 nm which is not observed in AX4 cells (Figure 7). We did not succeed in characterizing the metabolites that accumulate in *redA*-mutants despite many attempts.

CPR is a key enzyme in many metabolic processes, as a consequence of its close interactions with cytochrome P450 heme oxygenases. In particular, its participation in synthesis and/or degradation of important cellular compounds, such as retinoic acid, cholesterol and steroid hor-

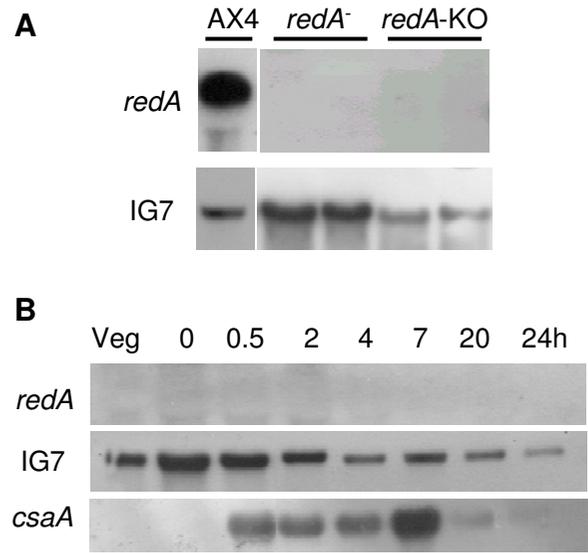


Figure 5
Disruption of *redA* results in cells lacking *redA* transcript. (A) Total RNA was prepared from exponentially growing AX4 cells (AX4) and from two independent clones of the mutants *redA*⁻ and *redA*-KO. Identical Northern blots were probed with *redA* and IG7 cDNAs as indicated. (B) Exponentially growing *redA*⁻ cells (Veg) were starved on filter pads and harvested at the indicated times (h) after starvation. Identical Northern blots of total RNA samples were probed with *redA*, IG7 and *csaA* cDNAs as indicated.

mones [2,29,30,35,36,54] may be related to abnormalities observed in development of organisms where CPR expression is abolished [28,32,55]. Development of homozygous mouse embryos carrying inactivating mutations in both alleles of the CPR gene is severely impaired, but lethality is only observed 10 to 13 days after zygote formation [28,29]. These findings indicate the importance of CPR in early animal development. Our results point to a role for a *D. discoideum* CPR in the metabolism of factors which control its cellular differentiation.

Conclusion

The *D. discoideum* genome encodes three genes (*redA*, *redB* and *redC*) for enzymes of diflavin oxidoreductases family. Disruption of *redA* led to mutant cells that form yellow mounds that fail to make proper fruiting bodies. The developmental arrest shown by this mutant implicates *redA*-encoded P450 oxidoreductase in the metabolism of compounds that control cell differentiation.

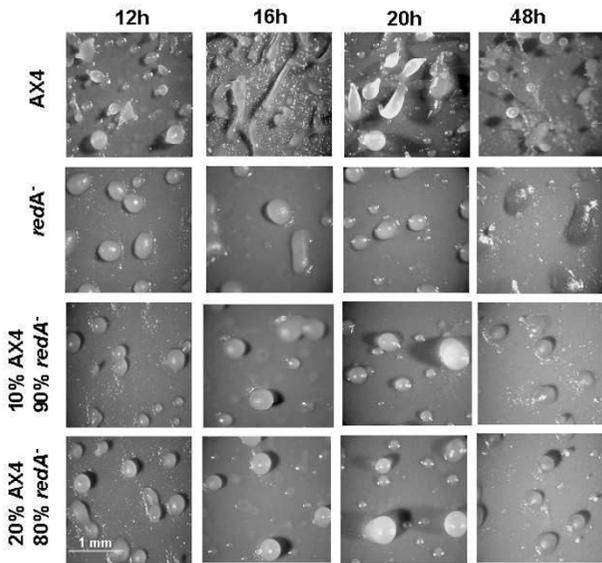


Figure 6
AX4 cells do not rescue redA⁻ phenotype. Exponentially growing redA⁻ and AX4 wild type cells were starved on filter pads mixed at the indicated proportions. At the indicated times (h) after starvation cells were photographed.

Methods

Cells and culture conditions

Dictyostelium discoideum strain AX4 and derived transformants were grown in axenic medium (HL-5) or in SM agar plates on lawns of *Klebsiella aerogenes* [56]. Complete and synchronous developmental program was undertaken by washing cells with 20 mM phosphate buffer (pH 6.4) and depositing them at 5×10^7 on nitrocellulose filters supported on buffer-saturated pads as previously described [56]. Strain DG1047 (redA⁻) was selected from a HindIII REMI-mutagenised library of strain AX4 selected for integration of the pBSR3 vector which carries the blasticidin S resistance cassette [34,57]. Morphological mutants were recognized by the structures formed within plaques generated by the blasticidin-resistant cells grown on SM agar in association with *K. aerogenes*.

Cloning of Dictyostelium redA cDNA

Regions flanking the plasmid insertion site in the REMI-mutant were isolated by plasmid rescue as described [34]. Genomic DNA from redA⁻ strain was digested with HindIII ligated and electroporated into *Escherichia coli* SURE cells (Stratagene). The rescue plasmid pRED was isolated from the ampicillin-resistant bacterial transformants and sequenced. A 2190 bp sequence partially encoding redA gene has been deposited in GenBank (access number AF012946). HindIII-linearized pRED was used to recapit-

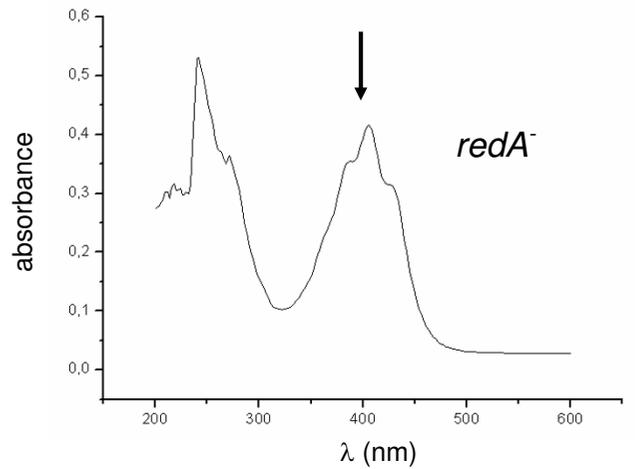
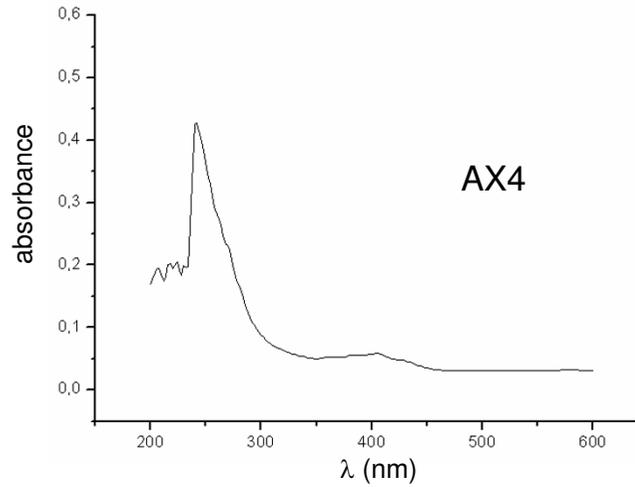


Figure 7
UV and visible spectra of the chloroform extracts from AX4 and redA⁻. Chloroform extracts of AX4 and redA⁻ cells collected after 48 hours starvation were analyzed using a UV/Visible spectrophotometer. The arrow points the absorption peak at 400 nm observed in the redA⁻ cell extract.

ulate redA mutation by homologous recombination as described [34].

A 1.8 kbp genomic fragment obtained from pRED by digestion with HindIII and SmaI was used as probe to screen a Lambda-ZAP (Stratagene) cDNA library derived from AX4 *D. discoideum* vegetative cells (kindly provided by Dr. Hudson Freeze, The Burnham Institute, La Jolla, USA). Screening of 200,000 plaques under high stringency conditions yielded twenty positives clones that were subjected to *in vivo* excision from a phagemid by transformation of *Escherichia coli* XL-1 blue MRF⁺ (Stratagene). The pBluescript SK clone (2B) with the largest cDNA insert was completely sequenced on both strands and the

sequence was deposited in GenBank (access number [DQ344637](#)).

Molecular cloning procedures were essentially as described [58], unless otherwise noted. DNA sequencing was performed on an ABI 377 automated sequencer (Perkin-Elmer).

Northern Blots

Total RNA was isolated from 5×10^7 *D. discoideum* cells at various developmental stages by using the Trizol (Invitrogen). Formaldehyde-agarose gel electrophoresis of RNA (20 µg) and transfers to nylon membranes (Amersham), were performed as described [58]. Probes were prepared with gel-purified DNA fragments radiolabeled with [α - 32 P]dATP and [α - 32 P]dCTP by the random hexanucleotide priming method (Random Primers DNA Labeling System, Invitrogen).

RT-qPCR

Reverse transcription was carried out with 5 µg of *D. discoideum* total RNA primed with a mixture of oligo dT and random hexamers using SuperScript First-Strand Synthesis System (Invitrogen). A 20 µg amount of the resulting cDNA were subjected to quantitative PCR using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) on a GeneAmp 5700 System (Applied Biosystems) using the default thermocycler program for all genes. Threshold values were normalized according to C_t of *D. discoideum* mitochondrial large subunit rRNA (IG7), which is expressed at similar levels throughout the *D. discoideum* development [59]. The fold change of each gene was calculated using the $2^{-\Delta C_t}$ method [60]. qPCR assays were performed in triplicate with the following gene-specific primer pairs: *redA* (5'-CCTATGGTGATGGTGTCCACCAAC-3' and 5'-CCCCACTAAATTGAATATGTGAAAGATTTAAACGA-3'), *redB* (5'-GCAACCGAAGAAGCAAACGAAGAATACAAT-3' and 5'-CAAAGGTTGAAGACCTGGGAAAGATTCTAA-3'), *redC* (5'-AGGTGGAGTCTTTGAAAGATGTTGTAATAATCC-3' and 5'-GGTCCAGGTACTGGTGTTCAC-3'), *ecmA* (5'-AGCTGATAGTTGCGATTCCA-3' and 5'-TACCTCCTGTACCACCACCA-3') and *mIA* (IG7) (5'-GTGGTTCCGGCACCTCGAT-3' and 5'-CACCCCAACCCTTGAAACT-3').

Chloroform extraction

5×10^8 AX4 and *redA*⁻ cells developed on filters for 48 hours were extracted with 1 ml chloroform and the organic phase was collected by centrifugation at $3000 \times g$ for 5 min at 4°C. UV/Visible spectra of the chloroform extracts were obtained in UV-2401PC Shimadzu spectrophotometer.

Sporulation efficiency assay

Mutants and wild-type cells were allowed to develop on nitrocellulose filters. At 0, 8, 12, 16, 20, 24 and 48 hours the cells were harvested from the filters with 20 mM phosphate buffer (pH 6.4) and sporulation efficiency was determined by detergent and heating treatment of the cells following plating on SM agar in association with *K. aerogenes* [56]. The number of plaques in the bacterial lawn indicated the number of viable spores. Wild-type AX4 cells submitted to 5-day starvation were used as a positive control for these experiments, to ensure recovery of fruiting bodies with viable spores.

List of abbreviations used

CPR, NADPH-cytochrome P450 oxidoreductase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.

Authors' contributions

DCGK carried out most of the experimental work and drafted the manuscript. LF performed RT-qPCR assays and helped with chloroform extractions. LCF was involved in the initial steps of this work and helped with cDNA cloning and sequence analyses. WFL isolated strain DG1047, participated in the design of the study and in writing the manuscript. AMDS coordinated the study, participated in its design and wrote the manuscript. All authors read and approved the final manuscript.

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References

1. Vermilion JL, Ballou DP, Massey V, Coon MJ: **Separate roles for FMN and FAD in catalysis by liver microsomal NADPH-cytochrome P-450 reductase.** *J Biol Chem* 1981, **256**:266-77.
2. Wang M, Roberts DL, Paschke R, Shea TM, Masters BS, Kim JJ: **Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes.** *Proc Natl Acad Sci USA* 1997, **94**:8411-6.
3. Guengerich FP: **Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity.** *Chem Res Toxicol* 2001, **14**:611-50.
4. Hasler JA: **Pharmacogenetics of cytochromes P450.** *Mol Aspects Med* 1999, **20**:12-24. 25-137
5. Porter TD: **An unusual yet strongly conserved flavoprotein reductase in bacteria and mammals.** *Trends Biochem Sci* 1991, **16**:154-8.
6. Murataliev MB, Feyereisen R, Walker FA: **Electron transfer by diflavin reductases.** *Biochim Biophys Acta* 2004, **1698**:1-26.
7. Olteanu H, Banerjee R: **Human methionine synthase reductase, a soluble P-450 reductase-like dual flavoprotein, is sufficient for NADPH-dependent methionine synthase activation.** *J Biol Chem* 2001, **276**:35558-63.
8. Paine MJ, Garner AP, Powell D, Sibbald J, Sales M, Pratt N, Smith T, Tew DG, Wolf CR: **Cloning and characterization of a novel human dual flavin reductase.** *J Biol Chem* 2000, **275**:1471-8.

9. Porter TD, Kasper CB: **NADPH-cytochrome P-450 oxidoreductase: flavin mononucleotide and flavin adenine dinucleotide domains evolved from different flavoproteins.** *Biochemistry* 1986, **25**:1682-7.
10. Schacter BA, Nelson EB, Marver HS, Masters BS: **Immunochemical evidence for an association of heme oxygenase with the microsomal electron transport system.** *J Biol Chem* 1972, **247**:3601-7.
11. Enoch HG, Strittmatter P: **Cytochrome b5 reduction by NADPH-cytochrome P-450 reductase.** *J Biol Chem* 1979, **254**:8976-81.
12. Ono T, Ozasa S, Hasegawa F, Imai Y: **Involvement of NADPH-cytochrome c reductase in the rat liver squalene epoxidase system.** *Biochim Biophys Acta* 1977, **486**:401-7.
13. Nelson DR: **Cytochrome P450 and the individuality of species.** *Arch Biochem Biophys* 1999, **369**:1-10.
14. Simmons DL, Lalley PA, Kasper CB: **Chromosomal assignments of genes coding for components of the mixed-function oxidase system in mice. Genetic localization of the cytochrome P-450PCN and P-450PB gene families and the NADPH-cytochrome P-450 oxidoreductase and epoxide hydratase genes.** *J Biol Chem* 1985, **260**:515-21.
15. Shephard EA, Phillips IR, Santisteban I, West LF, Palmer CN, Ashworth A, Povey S: **Isolation of a human cytochrome P-450 reductase cDNA clone and localization of the corresponding gene to chromosome 7q11.2.** *Ann Hum Genet* 1989, **53**(Pt 4):291-301.
16. Sutter TR, Loper JC: **Disruption of the *Saccharomyces cerevisiae* gene for NADPH-cytochrome P450 reductase causes increased sensitivity to ketoconazole.** *Biochem Biophys Res Commun* 1989, **160**:1257-66.
17. Hovemann BT, Sehlmeier F, Malz J: ***Drosophila melanogaster* NADPH-cytochrome P450 oxidoreductase: pronounced expression in antennae may be related to odorant clearance.** *Gene* 1997, **189**:213-9.
18. Yadav JS, Loper JC: **Cloning and characterization of the cytochrome P450 oxidoreductase gene from the zygomycete fungus *Cunninghamella*.** *Biochem Biophys Res Commun* 2000, **268**:345-53.
19. Porter TD, Kasper CB: **Coding nucleotide sequence of rat NADPH-cytochrome P-450 oxidoreductase cDNA and identification of flavin-binding domains.** *Proc Natl Acad Sci USA* 1985, **82**:973-7.
20. Malonek S, Rojas MC, Hedden P, Gaskin P, Hopkins P, Tudzynski B: **The NADPH-cytochrome P450 reductase gene from *Gibberella fujikuroi* is essential for gibberellin biosynthesis.** *J Biol Chem* 2004, **279**:25075-84.
21. Mizutani M, Ohta D: **Two isoforms of NADPH:cytochrome P450 reductase in *Arabidopsis thaliana*. Gene structure, heterologous expression in insect cells, and differential regulation.** *Plant Physiol* 1998, **116**:357-67.
22. Kunic B, Truan G, Breskvar K, Pompon D: **Functional cloning, based on azole resistance in *Saccharomyces cerevisiae*, and characterization of *Rhizopus nigricans* redox carriers that are differentially involved in the P450-dependent response to progesterone stress.** *Mol Genet Genomics* 2001, **265**:930-40.
23. Van Bogaert IN, Develter D, Soetaert W, Vandamme EJ: **Cloning and characterization of the NADPH cytochrome P450 reductase gene (CPR) from *Candida bombicola*.** *FEMS Yeast Res* 2007.
24. Venkateswarlu K, Lamb DC, Kelly DE, Manning NJ, Kelly SL: **The N-terminal membrane domain of yeast NADPH-cytochrome P450 (CYP) oxidoreductase is not required for catalytic activity in sterol biosynthesis or in reconstitution of CYP activity.** *J Biol Chem* 1998, **273**:4492-6.
25. Truan G, Epinat JC, Rougeulle C, Cullin C, Pompon D: **Cloning and characterization of a yeast cytochrome b5-encoding gene which suppresses ketoconazole hypersensitivity in a NADPH-P-450 reductase-deficient strain.** *Gene* 1994, **142**:123-7.
26. Lamb DC, Kelly DE, Manning NJ, Kaderbhai MA, Kelly SL: **Biodiversity of the P450 catalytic cycle: yeast cytochrome b5/NADH cytochrome b5 reductase complex efficiently drives the entire sterol 14-demethylation (CYP51) reaction.** *FEBS Lett* 1999, **462**:283-8.
27. Rogers KM, Pierson CA, Culbertson NT, Mo C, Sturm AM, Eckstein J, Barbuch R, Lees ND, Bard M: **Disruption of the *Candida albicans* CYB5 gene results in increased azole sensitivity.** *Antimicrob Agents Chemother* 2004, **48**:3425-35.
28. Shen AL, O'Leary KA, Kasper CB: **Association of multiple developmental defects and embryonic lethality with loss of microsomal NADPH-cytochrome P450 oxidoreductase.** *J Biol Chem* 2002, **277**:6536-41.
29. Otto DM, Henderson CJ, Carrie D, Davey M, Gundersen TE, Blomhoff R, Adams RH, Tickle C, Wolf CR: **Identification of novel roles of the cytochrome p450 system in early embryogenesis: effects on vasculogenesis and retinoic acid homeostasis.** *Mol Cell Biol* 2003, **23**:6103-16.
30. Ribes V, Otto DM, Dickmann L, Schmidt K, Schuhbaur B, Henderson C, Blomhoff R, Wolf CR, Tickle C, Dolle P: **Rescue of cytochrome P450 oxidoreductase (Por) mouse mutants reveals functions in vasculogenesis, brain and limb patterning linked to retinoic acid homeostasis.** *Dev Biol* 2007, **303**:66-81.
31. Keeney DS, Waterman MR: **Two novel sites of expression of NADPH cytochrome P450 reductase during murine embryogenesis: limb mesenchyme and developing olfactory neuroepithelia.** *Dev Dyn* 1999, **216**:511-7.
32. Horike N, Takemori H, Nonaka Y, Sonobe H, Okamoto M: **Molecular cloning of NADPH-cytochrome P450 oxidoreductase from silkworm eggs. Its involvement in 20-hydroxyecdysone biosynthesis during embryonic development.** *Eur J Biochem* 2000, **267**:6914-20.
33. Eichinger L, Pachebat JA, Glockner G, Rajandream MA, Sugang R, Berriman M, Song J, Olsen R, Szafranski K, Xu Q, et al.: **The genome of the social amoeba *Dictyostelium discoideum*.** *Nature* 2005, **435**:43-57.
34. Kuspa A, Loomis WF: **Tagging developmental genes in *Dictyostelium* by restriction enzyme-mediated integration of plasmid DNA.** *Proc Natl Acad Sci USA* 1992, **89**:8803-7.
35. Smith GC, Tew DG, Wolf CR: **Dissection of NADPH-cytochrome P450 oxidoreductase into distinct functional domains.** *Proc Natl Acad Sci USA* 1994, **91**:8710-4.
36. Dohr O, Paine MJ, Friedberg T, Roberts GC, Wolf CR: **Engineering of a functional human NADH-dependent cytochrome P450 system.** *Proc Natl Acad Sci USA* 2001, **98**:81-6.
37. Shen AL, Kasper CB: **Differential contributions of NADPH-cytochrome P450 oxidoreductase FAD binding site residues to flavin binding and catalysis.** *J Biol Chem* 2000, **275**:41087-91.
38. Sem DS, Kasper CB: **Interaction with arginine 597 of NADPH-cytochrome P-450 oxidoreductase is a primary source of the uniform binding energy used to discriminate between NADPH and NADH.** *Biochemistry* 1993, **32**:11548-58.
39. Nielsen H, Engelbrecht J, Brunak S, von Heijne G: **A neural network method for identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites.** *Int J Neural Syst* 1997, **8**:581-99.
40. Krogh A, Larsson B, von Heijne G, Sonnhammer EL: **Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes.** *J Mol Biol* 2001, **305**:567-80.
41. Ro DK, Ehltng J, Douglas CJ: **Cloning, functional expression, and subcellular localization of multiple NADPH-cytochrome P450 reductases from hybrid poplar.** *Plant Physiol* 2002, **130**:1837-51.
42. Tranbarger TJ, Forward BS, Misra S: **Regulation of NADPH-cytochrome P450 reductase expressed during Douglas-fir germination and seedling development.** *Plant Mol Biol* 2000, **44**:141-53.
43. He F, Chen YT: **Cloning and heterologous expression of the NADPH cytochrome P450 oxidoreductase genes from an industrial dicarboxylic acid-producing *Candida tropicalis*.** *Yeast* 2005, **22**:481-91.
44. Backes WL, Kelley RW: **Organization of multiple cytochrome P450s with NADPH-cytochrome P450 reductase in membranes.** *Pharmacol Ther* 2003, **98**:221-33.
45. van den Brink HJ, van Zeijl CM, Brons JF, van den Hondel CA, van Gorcum RF: **Cloning and characterization of the NADPH cytochrome P450 oxidoreductase gene from the filamentous fungus *Aspergillus niger*.** *DNA Cell Biol* 1995, **14**:719-29.

46. Benveniste I, Lesot A, Hasenfratz MP, Kochs G, Durst F: **Multiple forms of NADPH-cytochrome P450 reductase in higher plants.** *Biochem Biophys Res Commun* 1991, **177**:105-12.
47. Koopmann E, Hahlbrock K: **Differentially regulated NADPH:cytochrome P450 oxidoreductases in parsley.** *Proc Natl Acad Sci USA* 1997, **94**:14954-9.
48. Iranfar N, Fuller D, Loomis WF: **Genome-wide expression analyses of gene regulation during early development of *Dictyostelium discoideum*.** *Eukaryot Cell* 2003, **2**:664-70.
49. Ma PC, Siu CH: **A pharmacologically distinct cyclic AMP receptor is responsible for the regulation of gp80 expression in *Dictyostelium discoideum*.** *Mol Cell Biol* 1990, **10**:3297-306.
50. Nuckolls GH, Oshero N, Loomis WF, Spudich JA: **The *Dictyostelium* dual-specificity kinase *splA* is essential for spore differentiation.** *Development* 1996, **122**:3295-305.
51. O'Leary KA, Kasper CB: **Molecular basis for cell-specific regulation of the NADPH-cytochrome P450 oxidoreductase gene.** *Arch Biochem Biophys* 2000, **379**:97-108.
52. Simmons DL, Kasper CB: **Quantitation of mRNAs specific for the mixed-function oxidase system in rat liver and extrahepatic tissues during development.** *Arch Biochem Biophys* 1989, **271**:10-20.
53. Stromstedt M, Keeney DS, Waterman MR, Paria BC, Conley AJ, Dey SK: **Preimplantation mouse blastocysts fail to express CYP genes required for estrogen biosynthesis.** *Mol Reprod Dev* 1996, **43**:428-36.
54. Sevanian A, Nordenbrand K, Kim E, Ernster L, Hochstein P: **Micromosomal lipid peroxidation: the role of NADPH – cytochrome P450 reductase and cytochrome P450.** *Free Radic Biol Med* 1990, **8**:145-52.
55. Gonczy P, Echeverri C, Oegema K, Coulson A, Jones SJ, Copley RR, Duperon J, Oegema J, Brehm M, Cassin E, et al.: **Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III.** *Nature* 2000, **408**:331-6.
56. Sussman M: **Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions.** *Methods Cell Biol* 1987, **28**:9-29.
57. Shaulsky G, Kuspa A, Loomis WF: **A multidrug resistance transporter/serine protease gene is required for prestalk specialization in *Dictyostelium*.** *Genes Dev* 1995, **9**:1111-22.
58. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Struhl K: **Current Protocols in Molecular Biology.** New York: John Wiley & Sons, Inc; 1995.
59. Early AE, Williams JG: **A *Dictyostelium* prespore-specific gene is transcriptionally repressed by DIF in vitro.** *Development* 1988, **103**:519-24.
60. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.** *Methods* 2001, **25**:402-8.

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