Figure S1. Gene disruption of all members of the Dictyostelium Roco family. (A) Schematic drawing of roco4 gene disruption. A knockout construct was made by insertion of a bsr cassette in the BseJl site of the kinase domain of Roco4. Roman symbols refer to primer annealing sites for identification of correct integration events by PCR. (B) Identification of roco4-null cell line. gDNA was isolated from wild-type AX2 and potential knockout clones, and subjected to three PCR reactions. Primers I and II gave the expected products of 1169 and 2257 bp for AX2 and roco4-null respectively. Primers I and III and primers II and IV yielded no product for AX2 and bands of 451 and 862 bp for roco4-null respectively. (C) Identification of roco gene disruptions by PCR. Two primers that anneal just outside the knockout construct (Primers I and II for roco4) were used for PCR reactions with gDNA from wild-type AX2 and potential knockout clones as template. Clones with correct integration sites yield band shifts of around 1.1 kb, which is indicated in the figure.
Figure S2. Visualization of nuclei in *pats1-null*. Wild-type and *pats1*-null cells were fixed with paraformaldehyde and stained with DAPI to visualize nuclei. A substantial fraction of *pats1*-null cells is multinucleated, while wild-type cells are mostly mononucleated.
Figure S3. Differentiation of roco4-null in the presence of DIF. Exponentially growing cells in a 6-well plate were washed twice with phosphate buffer (PB) and incubated for 8 hours in PB+3mM cAMP at a density of $10^5$ cells/cm$^2$. After two washes with PB, the cells were incubated in PB with or without 100 nM DIF. After 16 hours, cells were inspected for vacuolization and pictures were taken. Both wild-type and roco4-null cells were able to vacuolize in the presence of DIF, as appointed by white arrows.
Figure S4. Sequence of the putative roco4 promoter. The starts of the promoter fragments are indicated as bold/underlined; a putative regulatory element starting at -783 is underlined/italic. The ATG start codon at position 1 is shown in capitals/bold/underlined. Translated amino acids are presented as single letters under the sequence between position 1-27.
Table SI. Primer sequences for expression of kinase domains and fabrication of KO-constructs.

| Primer          | Sequence (5’-3’)                                      |
|-----------------|------------------------------------------------------|
| Pats1kinasefw   | AGATCTAAAAATGACCTATGATGCAAATGTTAG                   |
| Pats1kinaserv   | CACTAGTAATTTTGATAAAATGAATACGAAAC                    |
| Roco4kinasefw   | GGGATCCAAAAATGTCAAATTTCCAGTGCTATCG                 |
| Roco4kinaserv   | CACTAGTACTACCATCACGCAATTTGAAAGAGGTGG               |
| Roco5kinasefw   | GAGATCTAAAAATGCTTGAAATCATTGAAAAGITGTGG             |
| Roco5kinaserv   | ACTAGTTGAAATTTGATTTAAAAACCAGTAGAGATTGAGAC         |
| Roco6kinasefw   | AGATCTAAAAATGCAACACAAGTAGATGAATC                  |
| Roco6kinaserv   | CACTAGTACTAAACACTGAAAACACCATTTACC                 |
| Roco7kinasefw   | GGATCCAAAAATGATCGATATCTATTCATTGGGC               |
| Roco7kinaserv   | CACTAGTATTATTTGATCTTCAATTTGAGTG                  |
| Roco8kinasefw   | GAGATCTAAAAATGAAATTTGTTTCCCTTTG                 |
| Roco8kinaserv   | CACTAGTTTTCGAAATTTGATATTGGAATCTTTAATTC          |
| Roco9fw         | CGGATCCAAAAATGACATCAATTGGCTAATTTTATTGG        |
| Roco9rv         | CTCTAATAAAAATTTGGAATTGATAAAC                  |
| Roco10kinasefw  | GAGATCTAAAAATGCCAAATTCGATCCTTTATTAG              |
| Roco10kinaserv  | TCTAGAGTTATATTACGACGCTAAAACGTCTTTAAAC         |
| Roco11kinasefw  | GGATCCAAAAATGATTTCAACTACCCAGTCCG                |
| Roco11kinaserv  | CACTAGTTTTCGAAATTTGGAATTTGTAACCTCC          |

The sequences contain restriction sites in **bold**, Kozak sequences in *italic* and start codons are **underlined**.

Table S2. Unique restriction sites for KO-constructs.

| Gene  | Site   |
|-------|--------|
| pats1 | Eco32I |
| roco4 | BseJ   |
| roco5 | SfuI  *
| roco6 | Eco105I |
| roco7 | BglII *
| roco8 | NdeI  *
| roco9 | BglII *
| roco10| SylI  *
| roco11| MfeI  *

Unique restriction sites were used to insert the bsr cassette. Asterisks refer to sticky sites that were made blunt for the construction of the KO-constructs.
### Table S3. Primer sequences for identification of correct integration events.

| Primer   | Sequence (5’-3’)                                                                 |
|----------|---------------------------------------------------------------------------------|
| Pats1kofw| GTGAGAATGCGGTAAAAGGCACTGGTTATCTCAAGTG                                             |
| Pats1korv| GGTGAAAGTTAGTAGTTATTTACTTGAGAGAGTTAGTG                                             |
| Roco4kofw| GTAGTGATTTATTCTGCACTTCCAAGATGGTTACACATC                                         |
| Roco4korv| CAAATGATCTAGATGGTATAAGCAATACCTACTACCAC                                          |
| Roco5kofw| CCTGAATTAATGGTGTATTGGTGTACAACTTTACCACCC                                          |
| Roco5korv| GTGGATGAGGTTGAGGTTGAGGTTGAGGTGTAACACTGCCAC                                         |
| Roco6kofw| GTGGTTGATGACAACCCACCATGCAACAGAGTGTAAAAG                                           |
| Roco6korv| GGTGTGTTTGTGTTGTTGTTGTTGATAATTGGAATGGTTGACTACCAC                                 |
| Roco7kofw| CACTAGGTCAAACAAATGTAATTTGTAAAGCACAACACTAGTG                                        |
| Roco7korv| GTGGTGTGTGGTGGGCGCTTGTGATTGTGAGGTG                                             |
| Roco8kofw| CTTTGCTTCATGTTGTTGCGAATGCTGTAAAAACACACCAC                                        |
| Roco8korv| CATATCTTACCTTAAATTTTAAACACTAAAGATCC                                             |
| Roco9kofw| CCAATCATTTGGTGTATTTAATGACACACACCTAC                                              |
| Roco9korv| GAAATAGGTTACACAAACACACACACACATAC                                               |
| Roco10kofw| GAAAAGTTGAAAATTTATGAGTGATTCAATTTTGTGTAAGATC                                      |
| Roco10korv| GATAAATGAACTGCAAACAGATGGTTAATGATGAGTCA                                          |
| Roco11kofw| TCACAATATTGGCTTGTTGAAAAATCCATTGTGATTG                                            |
| Roco11korv| GAGTAATAATGAAATCTATATTACCCACATATATAT                                              |

### Table S4. Primer sequences for RT-PCR.

| Primer   | Sequence (5’-3’)                                                                 |
|----------|---------------------------------------------------------------------------------|
| RTGbpCfw| CGTGAAATGAAACTGTTGCTAGACC                                                        |
| RTGbpCrV| CCAATAGAGGATGCAGTTAGATAACC                                                       |
| RTPats1fw| GATGGTTAGAGTTGATAATACC                                                          |
| RTPats1rv| CCAATAGGTTAAAATAATATAC                                                          |
| RTQkgAfw| GCAAGAGCAATGTCATATTAGGTG                                                        |
| RTQkgArv| GTATATCATTATTAACTACCATATCC                                                      |
| RTRoco4fw| CTCATGGTCCATAGTTTGTTGGAATG                                                       |
| RTRoco4rv| GGAATACCTTTGTTGAAATTCGGG                                                        |
| RTRoco5fw| GGAATACATTTACTGTAACCG                                                          |
| RTRoco3rv| CAGCTGGAAGAAAGACTACCCTAC                                                          |
| RTRoco6fw| GATACCCTGTTAGTTTGCTAGG                                                          |
| RTRoco6rv| CGTACCGATCCTTTATGATGACAC                                                     |
| RTRoco7fw| GTACAAGCTAACAATGTTCACTAG                                                       |
| RTRoco7rv| CCCATTATAATTACAGTGATCC                                                          |
| RTRoco8fw| GAAATCGGTGATTGGATATTG                                                           |
| RTRoco8rv| CAACAGCAGTTGATGATTTACTG                                                       |
| RTRoco9fw| CGTCAAGATTAATGTTTCTACATCC                                                          |
| RTRoco9rv| CCCAATACACACCATATCTGAG                                                          |
| RTRoco10fw| CGTTACCTGAAACAAATTATAAGTG                                                       |
| RTRoco10rv| CATACGGCTTCAGGTTGTTG                                                         |
| RTRoco11fw| CAATTATAACAAAAGCGGTGTAACCTG                                                       |
| RTRoco11rv| GCTAATTCGAAATGTAATATCC                                                          |
| RTIG7fw| TTACATTTATAGACCGAACACCAAGG                                                       |
| RTIG7rv| TTCCCTTTAGACCTATGGACCTTAGG                                                        |
Table S5. Primer sequences for expression cloning of Roco4, QkgA and Roco11.

| Primer   | Sequence (5'-3')                                                                 |
|----------|--------------------------------------------------------------------------------|
| Roco4fwA | CGGATCCAAATGGATCTCAACAAATTTAC                                               |
| Roco4rv1 | CTCAATGGTATTCTCTCAATAGATTACCAC                                               |
| Roco4fw2 | GAGTTAGATTTAAGTGATAATAAATATCAC                                               |
| Roco4rv2 | CCTATGAAACTAACCACAAAAACCTTTACC                                               |
| Roco4fw3 | GTGTTTGTATTCTCTCAAGATGTCATAACCACATC                                           |
| Roco4rvA | CACTGTAATGTTAGATTTAAGATGAAATGTGATAATC                                       |
| QkgAfw1  | GTACCTGAAACTCAATGACTGACTCCACTACT                                            |
| QkgAfw2  | GCAAGAGCATGTACATTAGGTG                                                      |
| QkgArv1  | ACTAGTAAATTGAGCAGGATAATTTTTTTAAAAATG                                         |
| QkgArvA  | ACCTAGTAAATTCGAGGATAATTTTTTTAAAAATG                                         |
| Roco11fwA| CTCTAGAATTGAGGAAACCATCAGATCAGATGAAATG                                    |
| Roco11rv1| CTTTATACCAGTACATTGGATCAAGATAC                                               |
| Roco11fw2| TCTGGGTTCTATCTGTTACCAATG                                                   |
| Roco11rv2| CGGAGCAATATAGTCAATACG                                                      |
| Roco11fw3| CAACAATCGATACACTATTACCG                                                    |
| Roco11rvA| GTCTAGATTTAGCAATTGTTAATTTTGGAACCTCC                                        |

The sequences contain BamHI (Roco4), BcuI (QkgA) and XbaI (Roco11) sites in **bold**, Kozak sequences in *italic* and start codons are *underlined*.

Table S6. Primer sequences for roco4 promoters.

| Primer   | Start bp | Sequence (5'-3')                                                                 |
|----------|----------|--------------------------------------------------------------------------------|
| Prom4fwA | -956     | CGGATCCAACGGTGCAAATAGTGTTGTCCTGTTAAAAAC                                         |
| Prom4fwB | -829     | CGGATCCACACATTGTTATGTAGT                                                   |
| Prom4fwC | -799     | CGGATCCACCAATGTTATGCTACGATTTACG                                           |
| Prom4fwD | -769     | CGGATCCACCAATGTTATGCTACGATTTACG                                           |
| Prom4fwE | -705     | CGGATCCACCAATGTTATGCTACGATTTACG                                           |
| Prom4fwF | -360     | CGGATCCACCAATGTTATGCTACGATTTACG                                           |
| Prom4fwG | -67      | CGGATCCACCAATGTTATGCTACGATTTACG                                           |

The sequences contain XhoI sites in **bold**.
Table S7. Locus tags for phylogenetic analysis of the deduced Roco proteins.

| Gene      | Dictyostelium discoideum | Dictyostelium purpureum | Dictyostelium fasciculatum | Polysphondylium pallidum |
|-----------|--------------------------|-------------------------|-----------------------------|--------------------------|
| gbpC/roco1| DDB0191359               | DPU_G0059624            | DFA_03461                   | PPL_12173                |
| qkgA/roco2| DDB0185215               | Not present             | Not present                 | Not present              |
| pats1/roco3| DDB0191503              | DPU_G0070698            | DFA_06290                   | PPL_08658                |
| roco4     | DDB0191509               | DPU_G0058498            | DFA_11519                   | PPL_09273                |
| roco5     | DDB0232931               | DPU_G0063182            | DFA_03850                   | PPL_10521                |
| roco6     | DDB0214834               | DPU_G0065240            | DFA_08323                   | PPL_12503                |
| roco7     | DDB0191295               | DPU_G0059300            | DFA_09719                   | PPL_05273                |
| roco8     | DDB0191480               | DPU_G0058976            | DFA_00686                   | PPL_04837                |
| roco9     | DDB0191512               | DPU_G0072160            | DFA_09477                   | PPL_07407                |
| roco10    | DDB0201665               | DPU_G0063892            | DFA_00911                   | PPL_02805                |
| roco11    | DDB0191297               | Not present             | Not present                 | Not present              |