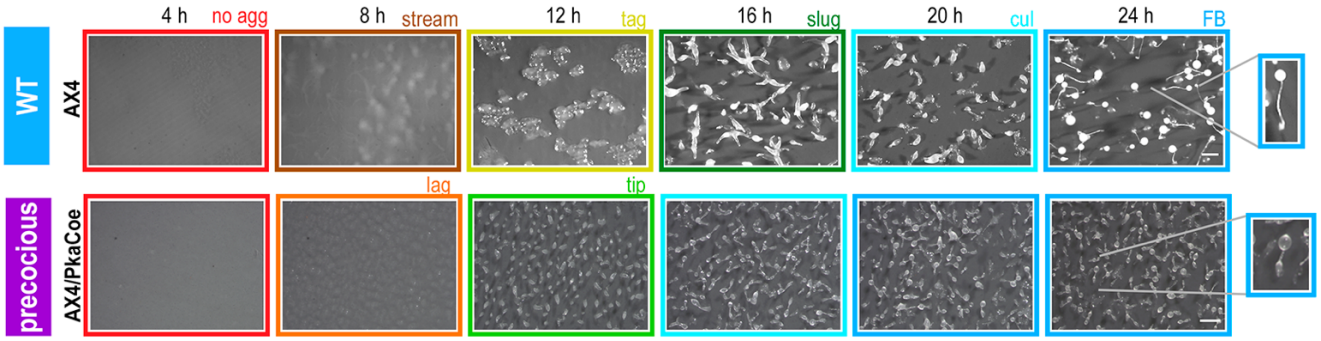
Transcriptional milestones in Dictyostelium development
Katoh-Kurasawa et al.

Supplemental materials

Supplemental Figures

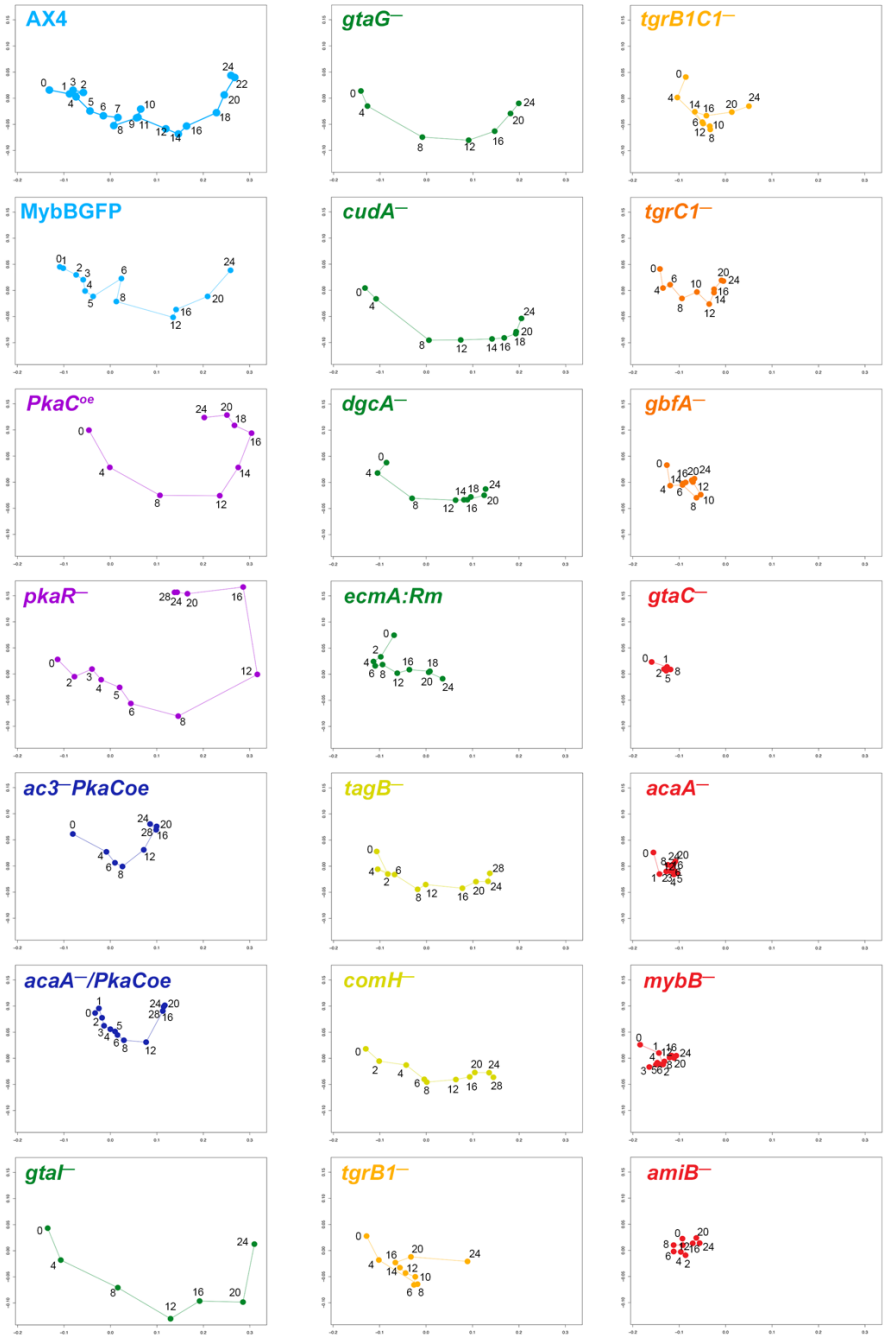
Supplemental_Fig_S1 Precocious culmination in the pkaC-overexpressor strain



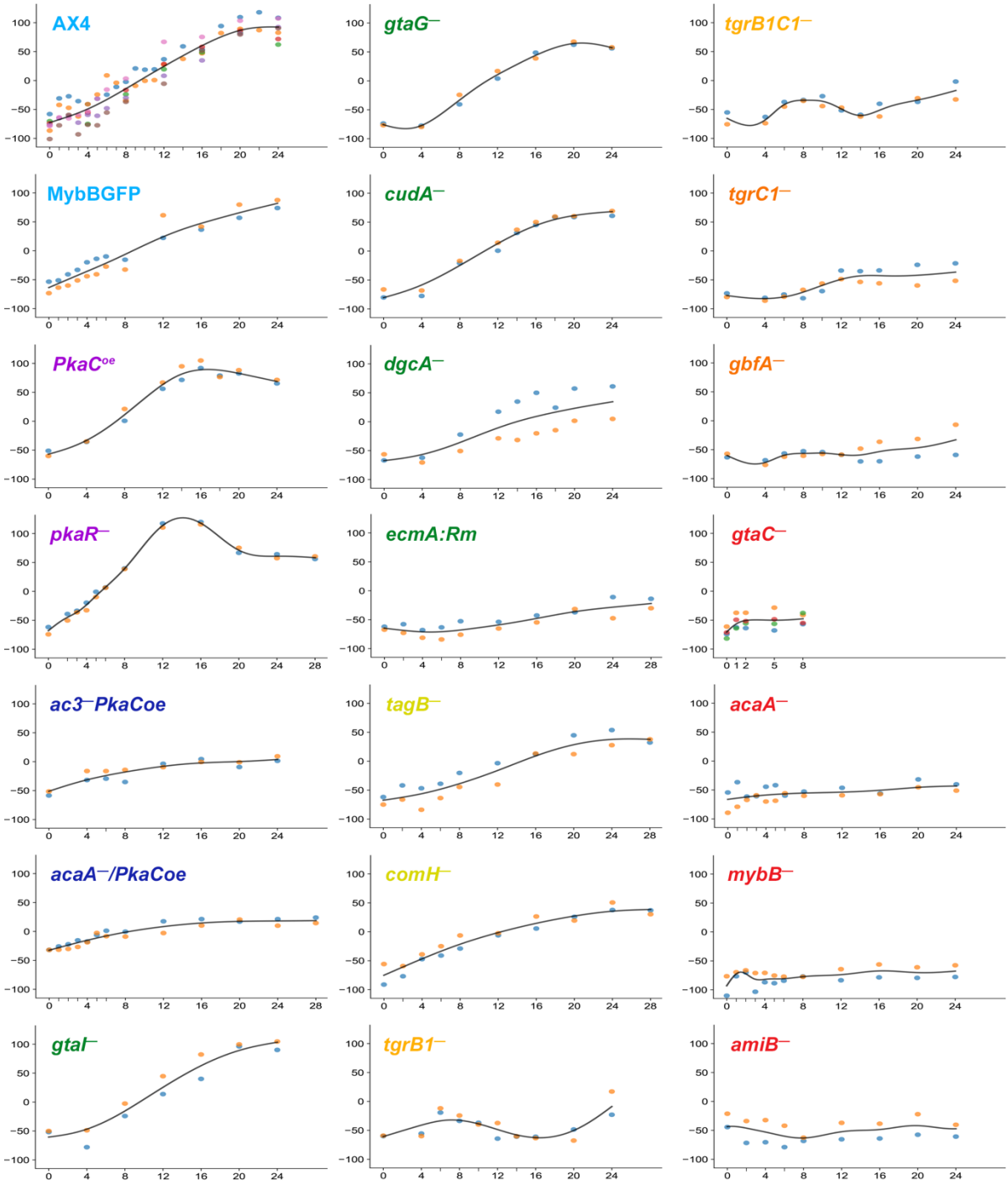
Supplemental_Fig_S1 Precocious culmination in the pkaC-overexpressor strain. Comparison between the wild type (AX4) and the *pkaC*^{oe} developmental morphologies. The AX4 images, all the experimental details, and the color codes are identical to those shown in Fig. 1 in the main text.

Supplemental_Fig_S2 MDS and PCA plots of individual strains

A.

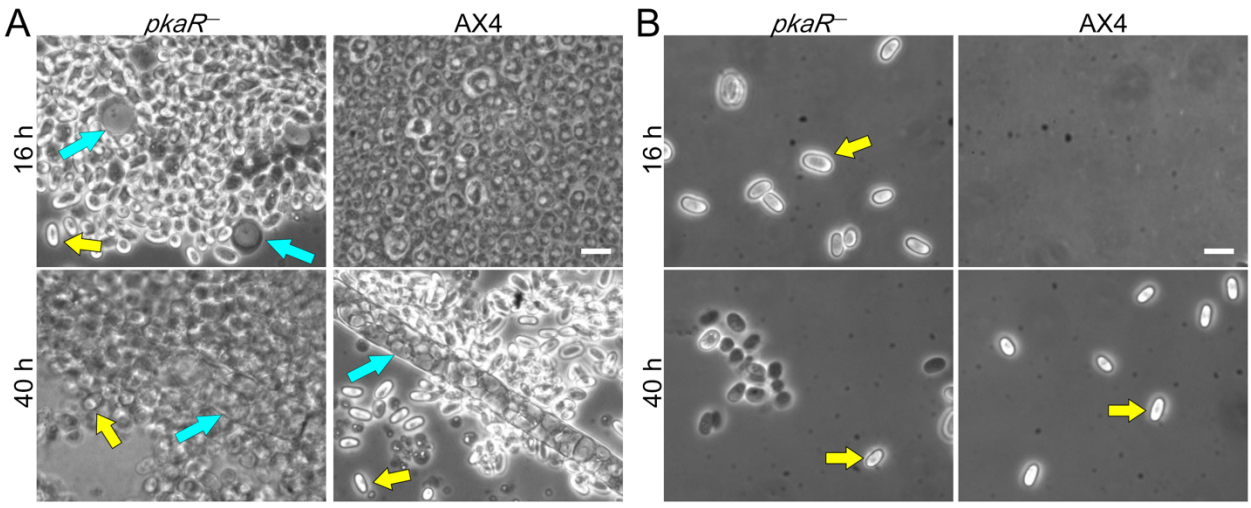


B.



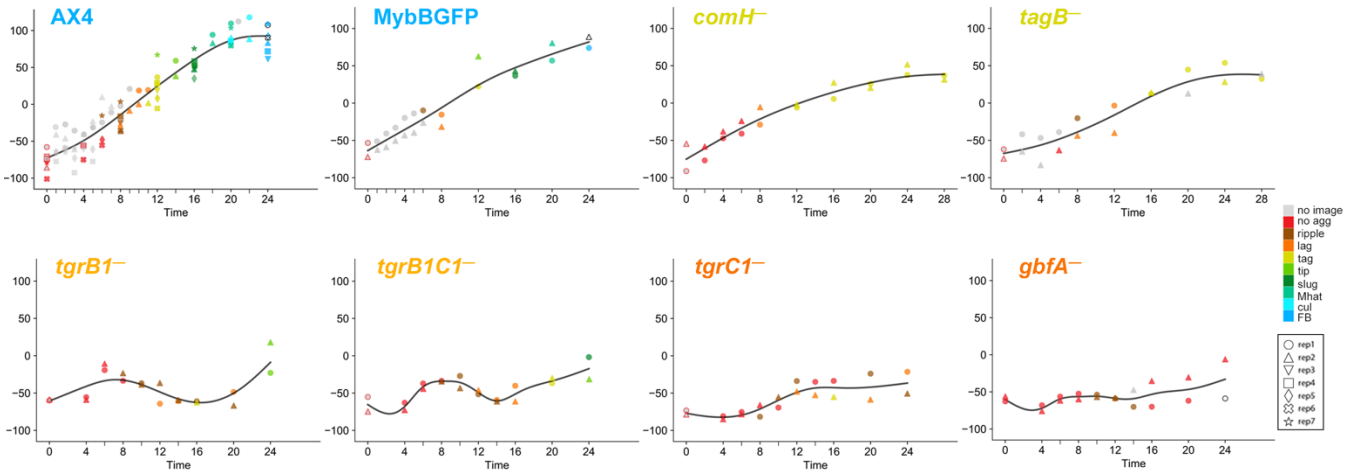
Supplemental_Fig_S2 MDS and PCA plots of individual strains. We analyzed the transcriptomes of the developing cells across time by RNA-seq and performed MDS (A) and PCA (B) using expression data from 2-7 replicates of each strain. The plots are identical to the respective analyses shown in aggregate in Figure 2, to provide better resolution. The PCA plots here include individual replicates of each strain plotted as different color circles to illustrate reproducibility.

Supplemental_Fig_S3 Precocious spore and stalk differentiation in the *pkaR*⁻ strain



Supplemental_Fig_S3 Precocious spore and stalk differentiation in the *pkaR*⁻ strain. We developed AX4 and *pkaR*⁻ cells on nitrocellulose filters for 16 h and 40 h, as indicated. (A) We placed whole mounds on microscope slides, squashed them gently under a cover-slip and examined the cell morphology with phase-contrast microscopy. Blue arrows indicate stalk cells (16 h) or stalk tubes (40 h) and yellow arrows indicate spores. Bars = 20 μm. (B) We treated the developing cells with detergent to eliminate amoebae and imaged them with phase-contrast microscopy. Yellow arrows indicate spores. Bars = 20 μm.

Supplemental_Fig_S4 Stage information on PCA plots



Supplemental_Fig_S4 Stage information on PCA plots. We added the representative stage information of each sample to the PC1 vs. time plots of the wild type, tight aggregate, and disaggregation phenotype groups (a subset of Supplemental_Fig_S2). PC1 (y-axis, arbitrary units) of each strain was plotted against time (x-axis, hours). The strain names are indicated in the plot and the color of the strain name represents the phenotype group: wild type (light blue), tight aggregate arrest (dark yellow), tight aggregate disaggregation (light orange), and loose aggregate disaggregation (dark orange). Representative morphological stages are plotted in different colors as indicated in the legend on the right. Samples at the zero time points were assigned a no agg stage. If the image was not captured for the samples at 0 h, red borders were added to gray symbols. White symbols with black outlines indicate samples for which representative stages were undetermined due to mixed morphologies.

Supplemental Files provided in this section

Supplemental_File_S1 Gene-set enrichment among milestone genes
Supplemental_File_S2 Gene-set enrichment among the regulons
Supplemental_File_S3 Gene-set enrichment in *tgr*-disaggregation and dedifferentiation
Supplemental_File_S4 Introductions to dictyExpress and Orange
Supplemental_File_S5 Computational methods
Supplemental_File_S6 Standard experimental methods

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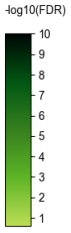
Supplemental_File_S1: Gene-set enrichment among milestone genes

All genes (except for non-expressed genes): 12,431 genes
Genes with Entrez ID (EID): 12,339 genes
Annotated genes: 4,510 genes (36.55%)

Milestone genes: 1,371 milestone genes at 8 stage transitions

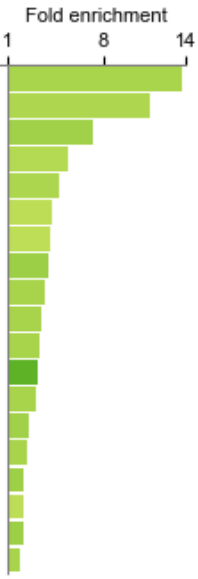
"Term": Enriched GO-term, KEGG-pathway, custom gene sets(Custom)
"Ontology": GO:Biological process(BP), Cellular component(CC), Molecular function(MF), KEGG:pathway(Path.), Custom
"Group": The number of genes with the term in the milestone gene set
"Reference": The number of genes with the term in the reference (with EID)
"FDR": false discovery rate, hypergeometric test
The bar graph size shows the fold enrichment and the color (see scale) represents the FDR.



Selected Enrichment terms: FDR ≤ 0.25, gene number in the group ≥ 2

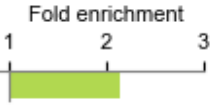
1) "no agg" to "ripple/stream" stage
Down-regulated:
all genes: 294, with EID: 292, term-annotated genes: **132 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
Ascorbate and aldarate metabolism	KEGG: Path.	2 (1.52%)	5 (0.11%)	7.65e-02
Lysine biosynthesis	KEGG: Path.	2 (1.52%)	6 (0.13%)	9.76e-02
Folate biosynthesis	KEGG: Path.	4 (3.03%)	19 (0.42%)	4.48e-02
translation factor activity	GO: MF	3 (2.27%)	19 (0.42%)	1.31e-01
Pyruvate metabolism	KEGG: Path.	4 (3.03%)	29 (0.64%)	8.36e-02
Glycine	KEGG: Path.	3 (2.27%)	24 (0.53%)	2.18e-01
Glutathione metabolism	KEGG: Path.	3 (2.27%)	25 (0.55%)	2.30e-01
lysosome	GO: CC	8 (6.06%)	69 (1.53%)	3.35e-02
methyltransferase activity	GO: MF	6 (4.55%)	56 (1.24%)	6.58e-02
homeostatic process	GO: BP	7 (5.30%)	70 (1.55%)	6.58e-02
cofactor metabolic process	GO: BP	7 (5.30%)	72 (1.60%)	6.58e-02
oxidoreductase activity	GO: MF	19 (14.39%)	207 (4.59%)	8.46e-04
peptidase activity	GO: MF	8 (6.06%)	91 (2.02%)	6.58e-02
lipid metabolic process	GO: BP	12 (9.09%)	159 (3.53%)	4.48e-02
extracellular region	GO: CC	11 (8.33%)	157 (3.48%)	6.58e-02
Biosynthesis of secondary metabolites	KEGG: Path.	18 (13.64%)	281 (6.23%)	3.78e-02
vacuole	GO: CC	8 (6.06%)	127 (2.82%)	2.18e-01
response to stress	GO: BP	22 (16.67%)	358 (7.94%)	3.35e-02
small molecule metabolic process	GO: BP	19 (14.39%)	353 (7.83%)	6.67e-02



Up-regulated:
all genes: 247, with EID: 246, term-annotated genes: **72 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
pst gene	Custom	17 (23.61%)	495 (10.98%)	1.09e-01



2) "ripple/stream" to "lag" stage
Down-regulated:
all genes: 1, with EID: 1, term-annotated genes: **0 genes**

Up-regulated:
all genes: 71, with EID: 70, term-annotated genes: **26 genes**
Enrichment annotation list: None

3) "lag" to "tag" stage
Down-regulated:
all genes: 11, with EID: 11, term-annotated genes: **2 genes**
Enrichment annotation list: None

Up-regulated:
all genes: 260, with EID: 259, term-annotated genes: **133 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
DNA replication	KEGG: Path.	21 (15.79%)	33 (0.73%)	3.41e-23
chromosome segregation	GO: BP	9 (6.77%)	18 (0.40%)	8.90e-09
Mismatch repair	KEGG: Path.	9 (6.77%)	19 (0.42%)	1.37e-08
mitotic nuclear division	GO: BP	6 (4.51%)	15 (0.33%)	1.91e-05
nuclear chromosome	GO: CC	6 (4.51%)	17 (0.38%)	3.81e-05
chromosome	GO: CC	22 (16.54%)	70 (1.55%)	4.34e-16
Base excision repair	KEGG: Path.	8 (6.02%)	28 (0.62%)	7.80e-06
Non-homologous end-joining	KEGG: Path.	2 (1.50%)	7 (0.16%)	6.65e-02
sig and sigN genes	Custom	16 (12.03%)	57 (1.26%)	6.10e-11
Nucleotide excision repair	KEGG: Path.	10 (7.52%)	37 (0.82%)	6.98e-07
chromosome organization	GO: BP	14 (10.53%)	74 (1.64%)	2.43e-07
Pyrimidine metabolism	KEGG: Path.	5 (3.76%)	29 (0.64%)	6.87e-03
chromatin/centromere	Custom	3 (2.26%)	18 (0.40%)	6.58e-02
cell cycle	GO: BP	26 (19.55%)	167 (3.70%)	2.69e-11
DNA metabolic process	GO: BP	15 (11.28%)	105 (2.33%)	2.89e-06
cAMP pulse induced	Custom	8 (6.02%)	56 (1.24%)	1.18e-03
microtubule organizing center	GO: CC	7 (5.26%)	50 (1.11%)	3.00e-03
cell division	GO: BP	14 (10.53%)	115 (2.55%)	3.81e-05
Homologous recombination	KEGG: Path.	3 (2.26%)	25 (0.55%)	1.28e-01
mitotic cell cycle	GO: BP	12 (9.02%)	100 (2.22%)	1.92e-04
helicase activity	GO: MF	5 (3.76%)	51 (1.13%)	6.65e-02
Ubiquitin mediated proteolysis	KEGG: Path.	5 (3.76%)	61 (1.35%)	1.22e-01
DNA binding	GO: MF	17 (12.78%)	221 (4.90%)	1.25e-03
ATPase activity	GO: MF	12 (9.02%)	168 (3.73%)	1.71e-02
cytoskeleton	GO: CC	11 (8.27%)	185 (4.10%)	7.64e-02
psp gene	Custom	17 (12.78%)	391 (8.67%)	2.25e-01

Fold enrichment: 1, 12, 22

4) “tag” to “tip” stage

Down-regulated:

all genes: 20, with EID: 20, term-annotated genes: **9 genes**

Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
cell adhesion	GO: BP	2 (22.22%)	83 (1.84%)	1.22e-01
plasma membrane	GO: CC	4 (44.44%)	268 (5.94%)	2.66e-02
cytoskeleton	GO: CC	2 (22.22%)	185 (4.10%)	1.74e-01
cytoskeleton organization	GO: BP	2 (22.22%)	196 (4.35%)	1.74e-01
vesicle-mediated transport	GO: BP	2 (22.22%)	233 (5.17%)	2.07e-01
signal transduction	GO: BP	3 (33.33%)	350 (7.76%)	1.73e-01

Fold enrichment: 1, 7, 13

Up-regulated:

all genes: 35, with EID: 35, term-annotated genes: **21 genes**

Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
57-aa protein family	Custom	10 (47.62%)	23 (0.51%)	4.13e-18
gtaG-dependent short protein	Custom	6 (28.57%)	49 (1.09%)	1.92e-07
cAMP pulse induced	Custom	2 (9.52%)	56 (1.24%)	5.47e-02
hssA/2C/7E family	Custom	3 (14.29%)	95 (2.11%)	2.29e-02
pst gene	Custom	13 (61.90%)	495 (10.98%)	1.28e-07
extracellular region	GO: CC	3 (14.29%)	157 (3.48%)	5.79e-02

Fold enrichment: 1, 48, 94

5) “tip” to “slug” stage

Down-regulated:

No selected genes.

Up-regulated:
all genes: 12, with EID: 12, term-annotated genes: **12 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR	Fold enrichment 1 19 36
hssA/2C/7E family	Custom	9 (75.00%)	95 (2.11%)	8.14e-13	

6) "slug" to "Mexican hat" stage
Down-regulated:
No selected genes.

Up-regulated:
all genes: 209, with EID: 209, term-annotated genes: **96 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR	Fold enrichment 1 3 5
gtaG-dependent short protein	Custom	5 (5.21%)	49 (1.09%)	1.06e-01	
psp gene	Custom	38 (39.58%)	391 (8.67%)	5.44e-15	
extracellular region	GO: CC	10 (10.42%)	157 (3.48%)	7.48e-02	

7) "Mexican hat" to "culmination" stage
Down-regulated:
all genes: 9, with EID: 9, term-annotated genes: **7 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR	Fold enrichment 1 8 14
hssA/2C/7E family	Custom	2 (28.57%)	95 (2.11%)	1.29e-02	
psp gene	Custom	5 (71.43%)	391 (8.67%)	2.60e-04	

Up-regulated:
all genes: 45, with EID: 45, term-annotated genes: **13 genes**
Enrichment annotation list:

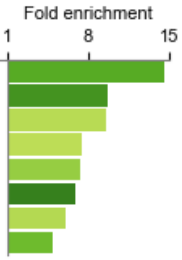
Term	Ontology	Group	Reference	FDR	Fold enrichment 1 15 29
gtaG-dependent short protein	Custom	4 (30.77%)	49 (1.09%)	2.13e-04	
sig and sigN genes	Custom	3 (23.08%)	57 (1.26%)	6.51e-03	

8) "culmination" to "fruiting body" stage
Down-regulated:
all genes: 3, with EID: 3, term-annotated genes: **2 genes**
Enrichment annotation list: None

Up-regulated:

all genes: 176, with EID: 175, term-annotated genes: **50 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
Starch and sucrose metabolism	KEGG: Path.	5 (10.00%)	31 (0.69%)	3.92e-04
anatomical structure formation involved in morphogenesis	GO: BP	8 (16.00%)	74 (1.64%)	3.48e-05
Fructose and mannose metabolism	KEGG: Path.	2 (4.00%)	19 (0.42%)	1.64e-01
transferase activity	GO: MF	2 (4.00%)	24 (0.53%)	2.24e-01
gtaG-dependent short protein	Custom	4 (8.00%)	49 (1.09%)	2.47e-02
extracellular region	GO: CC	12 (24.00%)	157 (3.48%)	5.07e-06
hydrolase activity	GO: MF	3 (6.00%)	45 (1.00%)	1.38e-01
cell differentiation	GO: BP	8 (16.00%)	145 (3.22%)	2.53e-03

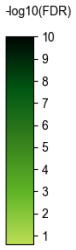


Supplemental_File_S2: Gene set enrichment among the regulons

All genes (except for non-expressed genes): 12,431 genes
Genes with Entrez ID (EID): 12,339 genes
Annotated genes: 4, 510 genes (36.55%)

Regulon clusters: 21 clusters containing 1099 selected genes

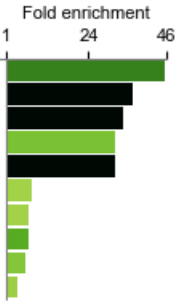
"Term": Enriched GO-term, KEGG-pathway, custom gene sets (Custom)
"Ontology": GO:Biological process(BP), Cellular component(CC), Molecular function(MF), KEGG:pathway(Path.), Custom
"Group": The number of genes with the term in the regulon cluster
"Reference": The number of genes with the term in the reference (with EID)
"FDR": false discovery rate, hypergeometric test
The bar graph size shows the fold enrichment and the color (see scale) represents the FDR.



Selected Enrichment terms: FDR ≤ 0.25, gene number in the group ≥ 2

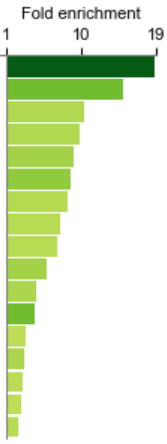
Cluster 1:
all genes: 30, with EID: 30, term-annotated genes: **22 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
ribonucleoprotein complex assembly	GO: BP	4 (18.18%)	18 (0.40%)	5.59e-06
Ribosome biogenesis in eukaryotes	KEGG: Path.	12 (54.55%)	68 (1.51%)	5.18e-16
ribosome biogenesis	GO: BP	10 (45.45%)	61 (1.35%)	3.25e-13
rRNA binding	GO: MF	2 (9.09%)	13 (0.29%)	5.15e-03
nucleolus	GO: CC	11 (50.00%)	72 (1.60%)	4.30e-14
helicase activity	GO: MF	2 (9.09%)	51 (1.13%)	5.01e-02
methyltransferase activity	GO: MF	2 (9.09%)	56 (1.24%)	5.37e-02
RNA binding	GO: MF	6 (27.27%)	169 (3.75%)	4.13e-04
protein-containing complex assembly	GO: BP	4 (18.18%)	133 (2.95%)	9.00e-03
cellular component assembly	GO: BP	4 (18.18%)	201 (4.46%)	3.35e-02



Cluster 2:
all genes: 66, with EID: 65, term-annotated genes: **40 genes**
Enrichment annotation list

Term	Ontology	Group	Reference	FDR
Oxidative phosphorylation	KEGG: Path.	9 (22.50%)	54 (1.20%)	3.49e-08
Citrate cycle (TCA cycle)	KEGG: Path.	4 (10.00%)	30 (0.67%)	2.94e-03
Various types of N-glycan biosynthesis	KEGG: Path.	2 (5.00%)	22 (0.49%)	1.16e-01
cell morphogenesis	GO: BP	2 (5.00%)	23 (0.51%)	1.16e-01
protein folding	GO: BP	3 (7.50%)	37 (0.82%)	5.03e-02
Phagosome	KEGG: Path.	4 (10.00%)	52 (1.15%)	1.77e-02
unfolded protein binding	GO: MF	2 (5.00%)	27 (0.60%)	1.43e-01
N-Glycan biosynthesis	KEGG: Path.	2 (5.00%)	30 (0.67%)	1.59e-01
Propanoate metabolism	KEGG: Path.	2 (5.00%)	32 (0.71%)	1.66e-01
generation of precursor metabolites and energy	GO: BP	4 (10.00%)	77 (1.71%)	5.03e-02
Ribosome	KEGG: Path.	4 (10.00%)	97 (2.15%)	8.51e-02
mitochondrion	GO: CC	9 (22.50%)	229 (5.08%)	2.94e-03
Carbon metabolism	KEGG: Path.	3 (7.50%)	102 (2.26%)	2.39e-01
cytoplasmic vesicle	GO: CC	6 (15.00%)	211 (4.68%)	8.51e-02
transmembrane transporter activity	GO: MF	4 (10.00%)	149 (3.30%)	1.86e-01
transmembrane transport	GO: BP	4 (10.00%)	164 (3.64%)	2.34e-01
Biosynthesis of secondary metabolites	KEGG: Path.	6 (15.00%)	281 (6.23%)	1.70e-01

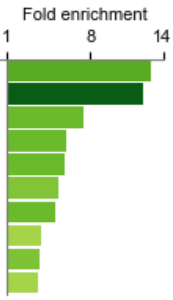


Cluster 3:
all genes: 68, with EID: 68, term-annotated genes: **66 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
ribosome	GO: CC	59 (89.39%)	93 (2.06%)	1.50e-100
structural constituent of ribosome	GO: MF	53 (80.30%)	85 (1.88%)	5.40e-87
Ribosome	KEGG: Path.	60 (90.91%)	97 (2.15%)	1.41e-101
rRNA binding	GO: MF	7 (10.61%)	13 (0.29%)	7.76e-10
structural molecule activity	GO: MF	53 (80.30%)	117 (2.59%)	1.08e-76
translation	GO: BP	52 (78.79%)	169 (3.75%)	4.18e-64
RNA binding	GO: MF	16 (24.24%)	169 (3.75%)	4.99e-09
ribosome biogenesis	GO: BP	3 (4.55%)	61 (1.35%)	1.84e-01
cytosol	GO: CC	8 (12.12%)	203 (4.50%)	3.12e-02

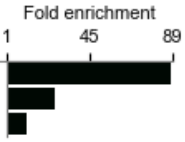
Cluster 4:
all genes: 55, with EID: 55, term-annotated genes: **23 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
Endocytosis	KEGG: Path.	5 (21.74%)	75 (1.66%)	4.29e-04
cytoskeletal protein binding	GO: MF	10 (43.48%)	158 (3.50%)	4.70e-08
protein-containing complex assembly	GO: BP	5 (21.74%)	133 (2.95%)	2.19e-03
cytoskeleton organization	GO: BP	6 (26.09%)	196 (4.35%)	2.19e-03
cellular component assembly	GO: BP	6 (26.09%)	201 (4.46%)	2.19e-03
cytoskeleton	GO: CC	5 (21.74%)	185 (4.10%)	7.34e-03
plasma membrane	GO: CC	7 (30.43%)	268 (5.94%)	2.19e-03
cytosol	GO: CC	4 (17.39%)	203 (4.50%)	5.80e-02
response to stress	GO: BP	7 (30.43%)	358 (7.94%)	6.23e-03
cytoplasmic vesicle	GO: CC	4 (17.39%)	211 (4.68%)	5.93e-02



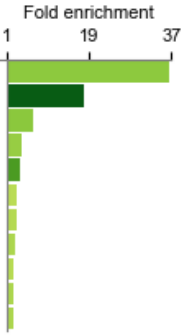
Cluster 5:
all genes: 41, with EID: 41, term-annotated genes: **40 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
Proteasome	KEGG: Path.	29 (72.50%)	37 (0.82%)	2.81e-57
peptidase activity	GO: MF	21 (52.50%)	91 (2.02%)	3.31e-25
catabolic process	GO: BP	25 (62.50%)	250 (5.54%)	2.40e-21



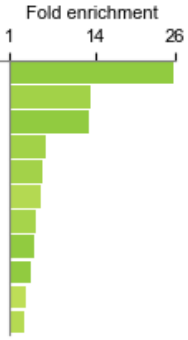
Cluster 6:
all genes: 74, with EID: 74, term-annotated genes: **41 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
Glycosaminoglycan degradation	KEGG: Path.	2 (4.88%)	6 (0.13%)	1.48e-02
cAMP pulse induced	Custom	9 (21.95%)	56 (1.24%)	4.65e-08
cell adhesion	GO: BP	5 (12.20%)	83 (1.84%)	1.39e-02
extracellular region	GO: CC	6 (14.63%)	157 (3.48%)	2.65e-02
anatomical structure development	GO: BP	15 (36.59%)	438 (9.71%)	7.49e-05
cell differentiation	GO: BP	4 (9.76%)	145 (3.22%)	1.88e-01
locomotion	GO: BP	5 (12.20%)	189 (4.19%)	1.50e-01
signal transduction	GO: BP	8 (19.51%)	350 (7.76%)	8.52e-02
plasma membrane	GO: CC	6 (14.63%)	268 (5.94%)	1.62e-01
response to stress	GO: BP	8 (19.51%)	358 (7.94%)	8.52e-02
pst gene	Custom	10 (24.39%)	495 (10.98%)	8.52e-02



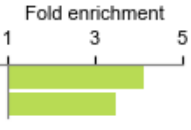
Cluster 7:
all genes: 40, with EID: 40, term-annotated genes: **14 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
cell death	GO: BP	2 (14.29%)	25 (0.55%)	1.93e-02
RNA degradation	KEGG: Path.	2 (14.29%)	48 (1.06%)	4.65e-02
anatomical structure formation involved in morphogenesis	GO: BP	3 (21.43%)	74 (1.64%)	1.93e-02
cell differentiation	GO: BP	3 (21.43%)	145 (3.22%)	4.65e-02
regulatory transcription factor	Custom	3 (21.43%)	160 (3.55%)	5.12e-02
chemotaxis	Custom	2 (14.29%)	110 (2.44%)	1.33e-01
locomotion	GO: BP	3 (21.43%)	189 (4.19%)	6.24e-02
kinase activity	GO: MF	5 (35.71%)	327 (7.25%)	1.93e-02
anatomical structure development	GO: BP	6 (42.86%)	438 (9.71%)	1.93e-02
cytoskeleton	GO: CC	2 (14.29%)	185 (4.10%)	2.36e-01
transcriptional regulation and chromatin organization	Custom	3 (21.43%)	289 (6.41%)	1.40e-01



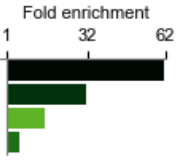
Cluster 8:
all genes: 23, with EID: 23, term-annotated genes: **10 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
kinase activity	GO: MF	3 (30.00%)	327 (7.25%)	1.71e-01
psp gene	Custom	3 (30.00%)	391 (8.67%)	1.71e-01



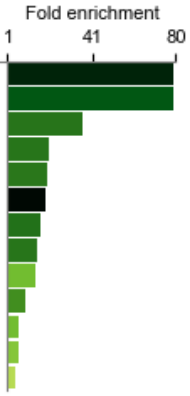
Cluster 9:
all genes: 20, with EID: 20, term-annotated genes: **18 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
sig and sigN genes	Custom	14 (77.78%)	57 (1.26%)	5.61e-24
cAMP pulse induced	Custom	7 (38.89%)	56 (1.24%)	1.77e-09
gtaG-dependent short protein	Custom	3 (16.67%)	49 (1.09%)	8.77e-04
pst gene	Custom	11 (61.11%)	495 (10.98%)	5.18e-07



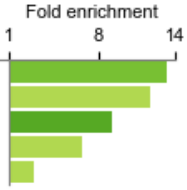
Cluster 10:
all genes: 36, with EID: 36, term-annotated genes: **19 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
chromosome segregation	GO: BP	6 (31.58%)	18 (0.40%)	6.51e-10
mitotic nuclear division	GO: BP	5 (26.32%)	15 (0.33%)	2.27e-08
DNA replication	KEGG: Path.	5 (26.32%)	33 (0.73%)	1.03e-06
chromosome	GO: CC	6 (31.58%)	70 (1.55%)	1.15e-06
chromosome organization	GO: BP	6 (31.58%)	74 (1.64%)	1.41e-06
cell cycle	GO: BP	13 (68.42%)	167 (3.70%)	1.07e-13
mitotic cell cycle	GO: BP	7 (36.84%)	100 (2.22%)	6.70e-07
cell division	GO: BP	7 (36.84%)	115 (2.55%)	1.15e-06
microtubule organizing center	GO: CC	3 (15.79%)	50 (1.11%)	3.09e-03
cytoskeleton	GO: CC	7 (36.84%)	185 (4.10%)	1.99e-05
cytoskeleton organization	GO: BP	5 (26.32%)	196 (4.35%)	3.09e-03
cytoskeletal protein binding	GO: MF	4 (21.05%)	158 (3.50%)	9.62e-03
DNA metabolic process	GO: BP	2 (10.53%)	105 (2.33%)	1.57e-01



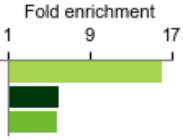
Cluster 11:
all genes: 77, with EID: 77, term-annotated genes: **24 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
cAMP pulse induced	Custom	4 (16.67%)	56 (1.24%)	4.62e-03
Starch and sucrose metabolism	KEGG: Path.	2 (8.33%)	31 (0.69%)	1.13e-01
cell differentiation	GO: BP	7 (29.17%)	145 (3.22%)	3.29e-04
cell adhesion	GO: BP	3 (12.50%)	83 (1.84%)	1.13e-01
anatomical structure development	GO: BP	7 (29.17%)	438 (9.71%)	1.01e-01



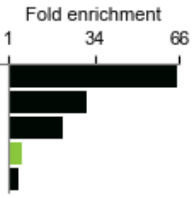
Cluster 12:
all genes: 57, with EID: 57, term-annotated genes: **35 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
cell-cell signaling	GO: BP	2 (5.71%)	16 (0.35%)	7.65e-02
psp gene	Custom	18 (51.43%)	391 (8.67%)	2.10e-09
extracellular region	GO: CC	7 (20.00%)	157 (3.48%)	2.80e-03



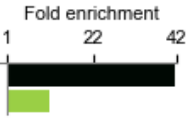
Cluster 13:
all genes: 64, with EID: 64, term-annotated genes: **51 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
57-aa protein family	Custom	17 (33.33%)	23 (0.51%)	7.13e-29
gtaG-dependent short protein	Custom	17 (33.33%)	49 (1.09%)	1.27e-21
hssA/2C/7E family	Custom	23 (45.10%)	95 (2.11%)	1.84e-25
cAMP pulse induced	Custom	4 (7.84%)	56 (1.24%)	1.24e-02
pst gene	Custom	28 (54.90%)	495 (10.98%)	5.22e-14



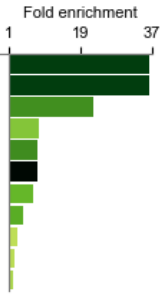
Cluster 14:
all genes: 19, with EID: 19, term-annotated genes: **16 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
hssA/2C/7E family	Custom	14 (87.50%)	95 (2.11%)	7.32e-22
gtaG-dependent short protein	Custom	2 (12.50%)	49 (1.09%)	3.15e-02



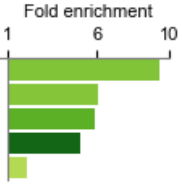
Cluster 15:
all genes: 91, with EID: 91, term-annotated genes: **67 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
cell wall	GO: CC	7 (10.45%)	13 (0.29%)	4.08e-09
external encapsulating structure	GO: CC	7 (10.45%)	13 (0.29%)	4.08e-09
cell wall organization or biogenesis	GO: BP	5 (7.46%)	15 (0.33%)	2.20e-05
Starch and sucrose metabolism	KEGG: Path.	4 (5.97%)	31 (0.69%)	8.54e-03
anatomical structure formation involved in morphogenesis	GO: BP	9 (13.43%)	74 (1.64%)	1.73e-05
psp gene	Custom	47 (70.15%)	391 (8.67%)	6.90e-34
cAMP pulse induced	Custom	6 (8.96%)	56 (1.24%)	1.46e-03
cell differentiation	GO: BP	10 (14.93%)	145 (3.22%)	4.91e-04
carbohydrate metabolic process	GO: BP	4 (5.97%)	88 (1.95%)	2.45e-01
extracellular region	GO: CC	6 (8.96%)	157 (3.48%)	1.85e-01
anatomical structure development	GO: BP	13 (19.40%)	438 (9.71%)	8.03e-02



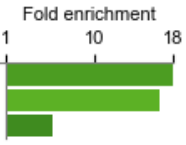
Cluster 16:
all genes: 74, with EID: 74, term-annotated: **39 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
gtaG-dependent short protein	Custom	4 (10.26%)	49 (1.09%)	7.65e-03
hssA/2C/7E family	Custom	5 (12.82%)	95 (2.11%)	9.14e-03
extracellular region	GO: CC	8 (20.51%)	157 (3.48%)	6.64e-04
psp gene	Custom	17 (43.59%)	391 (8.67%)	1.62e-07
pst gene	Custom	9 (23.08%)	495 (10.98%)	1.35e-01



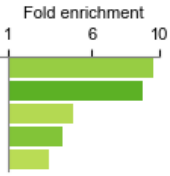
Cluster 17:
all genes: 61, with EID: 59, term-annotated genes: **22 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
sig and sigN genes	Custom	5 (22.73%)	57 (1.26%)	9.05e-05
gtaG-dependent short protein	Custom	4 (18.18%)	49 (1.09%)	7.79e-04
psp gene	Custom	11 (50.00%)	391 (8.67%)	1.60e-05



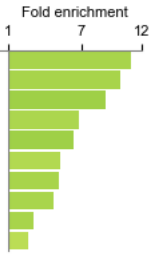
Cluster 18:
all genes: 46, with EID: 45, term-annotated genes: **19 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
anatomical structure formation involved in morphogenesis	GO: BP	3 (15.79%)	74 (1.64%)	2.72e-02
extracellular region	GO: CC	6 (31.58%)	157 (3.48%)	7.23e-04
cell differentiation	GO: BP	3 (15.79%)	145 (3.22%)	1.30e-01
psp gene	Custom	7 (36.84%)	391 (8.67%)	8.41e-03
oxidoreductase activity	GO: MF	3 (15.79%)	207 (4.59%)	1.84e-01



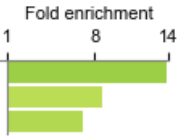
Cluster 19:
all genes: 58, with EID: 58, term-annotated genes: **18 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
hydrolase activity	GO: MF	2 (11.11%)	45 (1.00%)	6.52e-02
gtaG-dependent short protein	Custom	2 (11.11%)	49 (1.09%)	6.52e-02
cell adhesion	GO: BP	3 (16.67%)	83 (1.84%)	4.22e-02
anatomical structure formation involved in morphogenesis	GO: BP	2 (11.11%)	74 (1.64%)	9.00e-02
extracellular region	GO: CC	4 (22.22%)	157 (3.48%)	4.22e-02
hssA/2C/7E family	Custom	2 (11.11%)	95 (2.11%)	1.26e-01
cell differentiation	GO: BP	3 (16.67%)	145 (3.22%)	6.52e-02
regulatory transcription factor	Custom	3 (16.67%)	160 (3.55%)	7.25e-02
pst gene	Custom	6 (33.33%)	495 (10.98%)	6.52e-02
transcriptional regulation and chromatin organization	Custom	3 (16.67%)	289 (6.41%)	1.99e-01



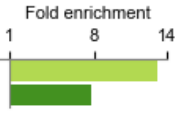
Cluster 20:
all genes: 54, with EID: 54, term-annotated genes: **20 genes**
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
gtaG-dependent short protein	Custom	3 (15.00%)	49 (1.09%)	3.50e-02
Phagosome	KEGG: Path.	2 (10.00%)	52 (1.15%)	2.10e-01
hssA/2C/7E family	Custom	3 (15.00%)	95 (2.11%)	1.15e-01



Cluster 21:
all genes: 45, with EID: 45, term-annotated genes: **12 genes**
Enrichment annotation list:

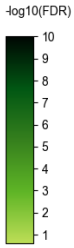
Term	Ontology	Group	Reference	FDR
sig and sigN genes	Custom	2 (16.67%)	57 (1.26%)	1.24e-01
psp gene	Custom	8 (66.67%)	391 (8.67%)	2.81e-05



Supplemental_File_S3: Gene-set enrichment in *tgr*-disaggregation and dedifferentiation

All genes (except for non-expressed genes): 12,431 genes
Genes with Entrez ID (EID): 12,339 genes
Annotated genes: 4,510 genes (36.55%)

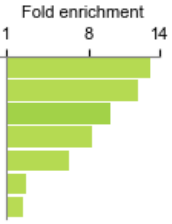
"Term": Enriched GO-term, KEGG-pathway, custom gene sets (Custom)
"Ontology": GO:Biological process(BP), Cellular component(CC), Molecular function(MF), KEGG:pathway(Path.), Custom
"Group": The number of genes with the term in the disagg gene set
"Reference": The number of genes with the term in the reference (with EID)
"FDR": false discovery rate, hypergeometric test
The bar graph size shows the fold enrichment and the color (see scale) represents the FDR.



Selected Enrichment terms: FDR ≤ 0.25, gene number in the group ≥ 2

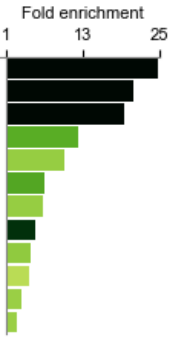
***tgr*-disagg 8 hr vs 6hr (padj ≤ 0.01, FoldChange ≥ 2.5)**
all genes: 72, with EID: 70, term-annotated genes: **31 genes** (44.0%)
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
Lysine degradation	KEGG: Path.	2 (6.45%)	22 (0.49%)	1.47e-01
Tryptophan metabolism	KEGG: Path.	2 (6.45%)	24 (0.53%)	1.47e-01
lipid binding	GO: MF	4 (12.90%)	59 (1.31%)	4.79e-02
membrane organization	GO: BP	3 (9.68%)	53 (1.18%)	1.47e-01
homeostatic process	GO: BP	3 (9.68%)	70 (1.55%)	1.47e-01
anatomical structure development	GO: BP	8 (25.81%)	438 (9.71%)	1.47e-01
pst gene	Custom	8 (25.81%)	495 (10.98%)	1.71e-01



***tgr*-disagg 12hr vs 8hr (padj ≤ 0.01, FoldChange ≥ 2.5)**
all genes: 218, with EID: 217, term-annotated genes: **102 genes** (47.0%)
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
ribosome biogenesis	GO: BP	34 (33.33%)	61 (1.35%)	2.18e-40
nucleolus	GO: CC	34 (33.33%)	72 (1.60%)	2.32e-37
Ribosome biogenesis in eukaryotes	KEGG: Path.	30 (29.41%)	68 (1.51%)	1.10e-31
ribonucleoprotein complex assembly	GO: BP	5 (4.90%)	18 (0.40%)	4.97e-04
rRNA binding	GO: MF	3 (2.94%)	13 (0.29%)	2.48e-02
helicase activity	GO: MF	8 (7.84%)	51 (1.13%)	2.43e-04
nucleocytoplasmic transport	GO: BP	4 (3.92%)	26 (0.58%)	2.48e-02
RNA binding	GO: MF	21 (20.59%)	169 (3.75%)	1.57e-09
methyltransferase activity	GO: MF	6 (5.88%)	56 (1.24%)	1.76e-02
nucleotidyltransferase activity	GO: MF	3 (2.94%)	29 (0.64%)	1.83e-01
nucleoplasm	GO: CC	7 (6.86%)	91 (2.02%)	3.23e-02
ATPase activity	GO: MF	10 (9.80%)	168 (3.73%)	3.23e-02



dediff_medium 0.5-2 hr vs buf allT (padj ≤ 0.01, FoldChange ≥ 4)
all genes: 360, with EID: 359, term-annotated genes: **162 genes** (45.0%)
Enrichment annotation list:

Term	Ontology	Group	Reference	FDR
Ascorbate and aldarate metabolism	KEGG: Path.	2 (1.23%)	5 (0.11%)	1.03e-01
Pentose and glucuronate interconversions	KEGG: Path.	2 (1.23%)	6 (0.13%)	1.42e-01
Ribosome biogenesis in eukaryotes	KEGG: Path.	17 (10.49%)	68 (1.51%)	1.61e-08
Purine metabolism	KEGG: Path.	10 (6.17%)	50 (1.11%)	2.35e-04
nucleolus	GO: CC	13 (8.02%)	72 (1.60%)	8.41e-05
ribosome biogenesis	GO: BP	11 (6.79%)	61 (1.35%)	2.35e-04
Alanine	KEGG: Path.	4 (2.47%)	23 (0.51%)	9.26e-02
methyltransferase activity	GO: MF	9 (5.56%)	56 (1.24%)	3.44e-03
RNA polymerase	KEGG: Path.	4 (2.47%)	25 (0.55%)	1.03e-01
Folate biosynthesis	KEGG: Path.	3 (1.85%)	19 (0.42%)	1.92e-01
2-Oxocarboxylic acid metabolism	KEGG: Path.	3 (1.85%)	19 (0.42%)	1.92e-01
Pyrimidine metabolism	KEGG: Path.	4 (2.47%)	29 (0.64%)	1.45e-01
Biosynthesis of amino acids	KEGG: Path.	8 (4.94%)	62 (1.37%)	2.20e-02
isomerase activity	GO: MF	6 (3.70%)	50 (1.11%)	9.26e-02
homeostatic process	GO: BP	8 (4.94%)	70 (1.55%)	4.38e-02
lyase activity	GO: MF	7 (4.32%)	66 (1.46%)	9.26e-02
Carbon metabolism	KEGG: Path.	9 (5.56%)	102 (2.26%)	1.02e-01
small molecule metabolic process	GO: BP	31 (19.14%)	353 (7.83%)	8.91e-05
ligase activity	GO: MF	7 (4.32%)	80 (1.77%)	1.74e-01
oxidoreductase activity	GO: MF	18 (11.11%)	207 (4.59%)	7.92e-03
mitochondrion	GO: CC	18 (11.11%)	229 (5.08%)	2.05e-02
Biosynthesis of secondary metabolites	KEGG: Path.	21 (12.96%)	281 (6.23%)	1.74e-02
cellular amino acid metabolic process	GO: BP	9 (5.56%)	124 (2.75%)	2.11e-01

	disaggregation_8to12		dedifferentiation	
	gene #	/list	gene #	/list
Milestone_noAgg_down (294 genes)	10	3%	48	16%
Regulon 1 (30 genes)	30	100%	15	50%
U3 related_rRNA processing (56 genes)	45	80%	21	38%
G-protein-coupled receptor (28 genes)	4	14.3%	1	3.6%
Overlap between two sets	70	19%	70	32%

DE on data from Nichols, et al. (2020): dediff_set
dediff_set overlap with all genes: 2.9%

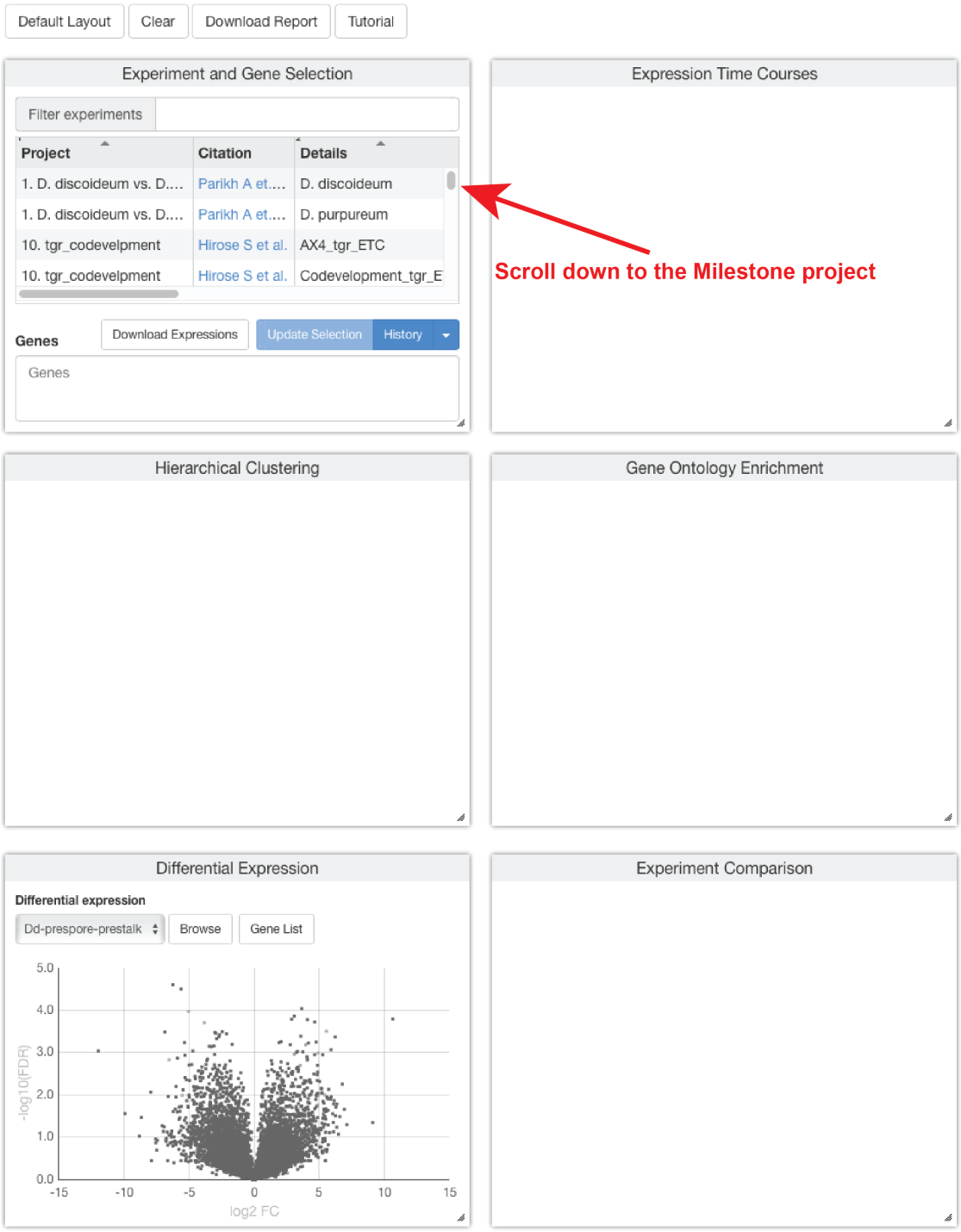
overlap between disaggregation_8to12 group and dediff_set: 32.1%
p-val: 9.25E-55

overlap between disaggregation_6to8 group and dediff_set: 2.8%
p-val: 6.21E-01

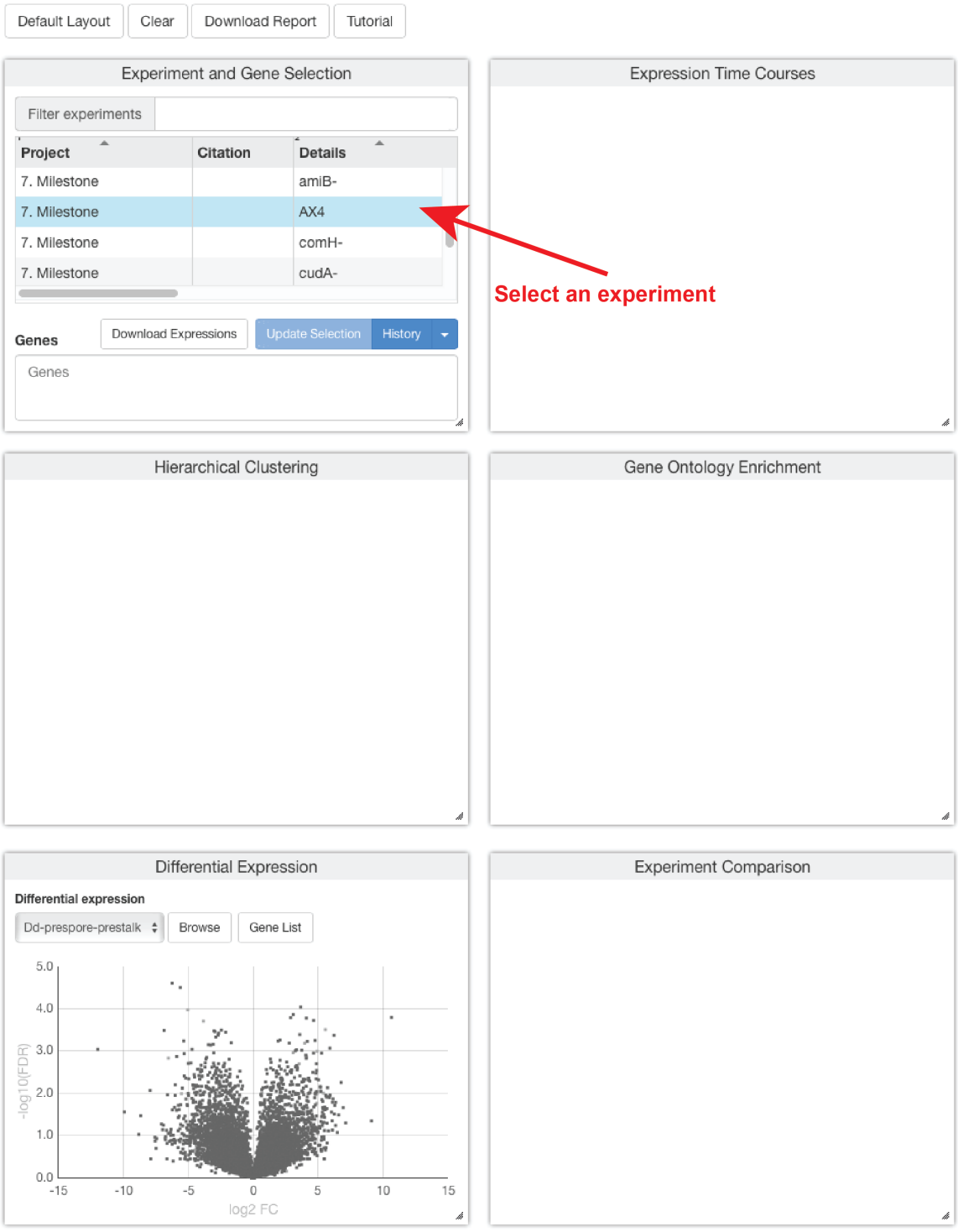
Supplemental_File_S4 Introduction to data mining in dictyExpress and Orange

A. An introduction to dictyExpress

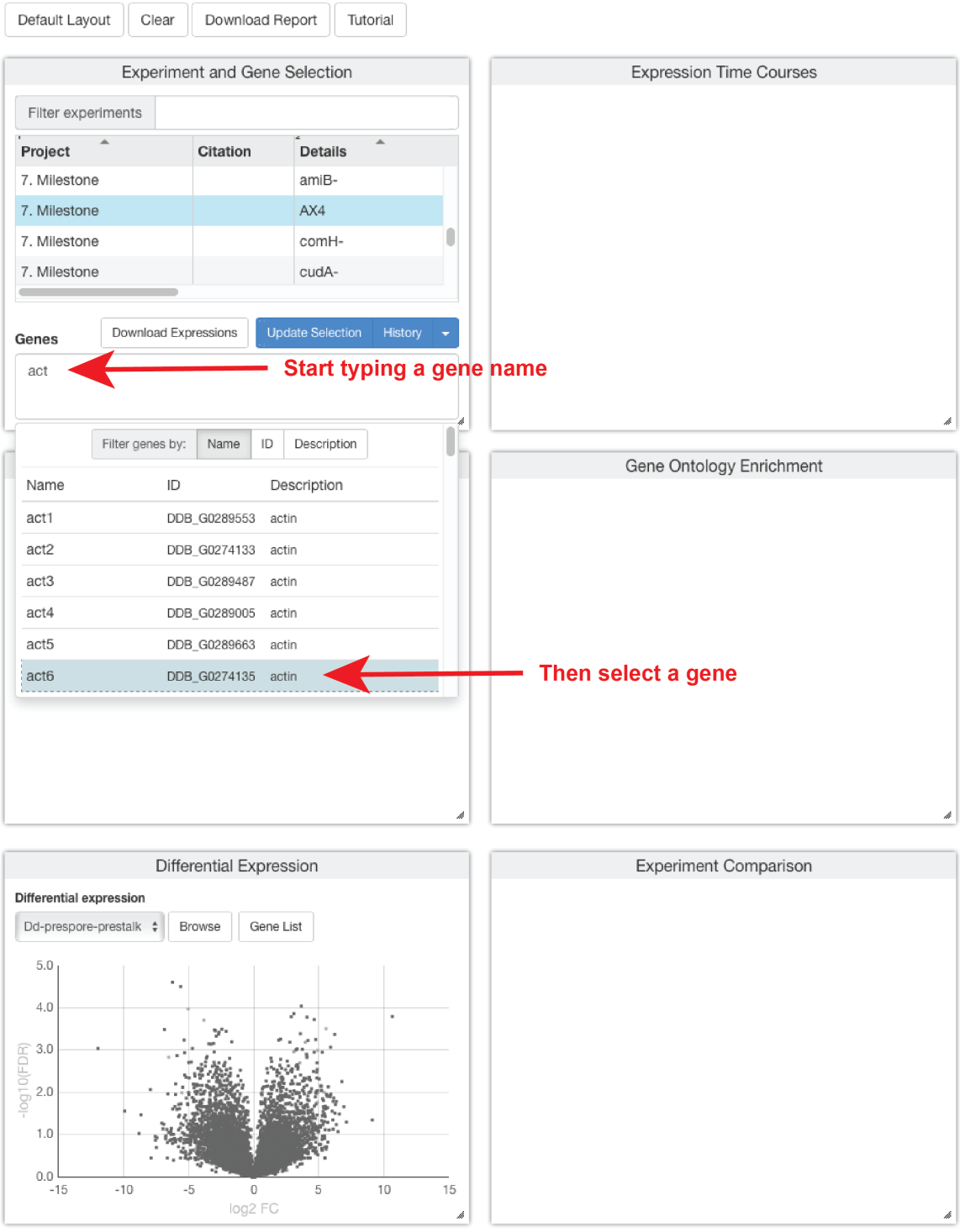
Open dictyExpress in a web browser: https://dictyexpress.research.bcm.edu, and press “Run dictyExpress”. If you are new to dictyExpress, follow the brief online tutorial before you proceed with the following suggestion.



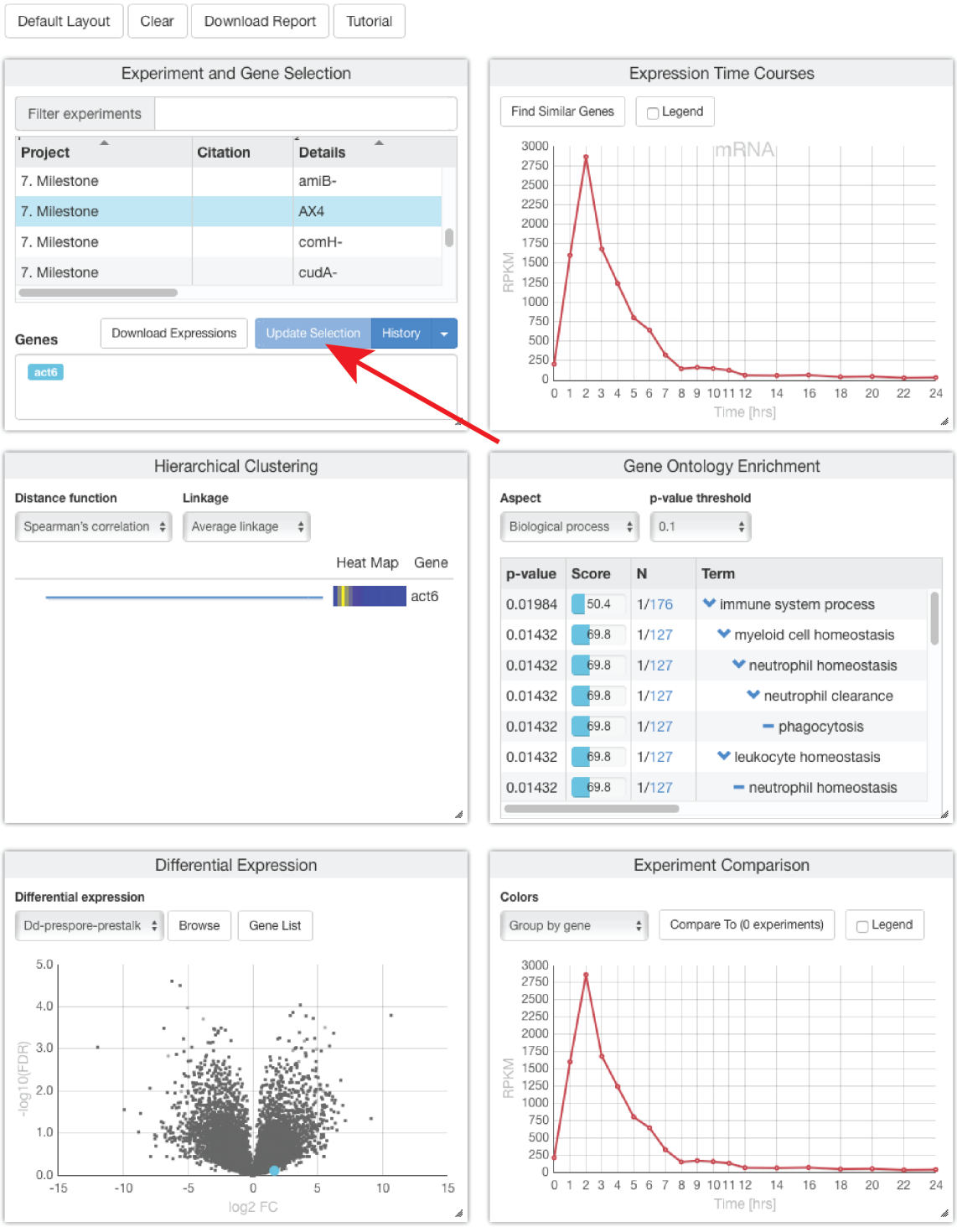
Select one of the Milestone project experiments in the ‘Experiment and Gene Selection’ panel. We recommend starting with AX4.



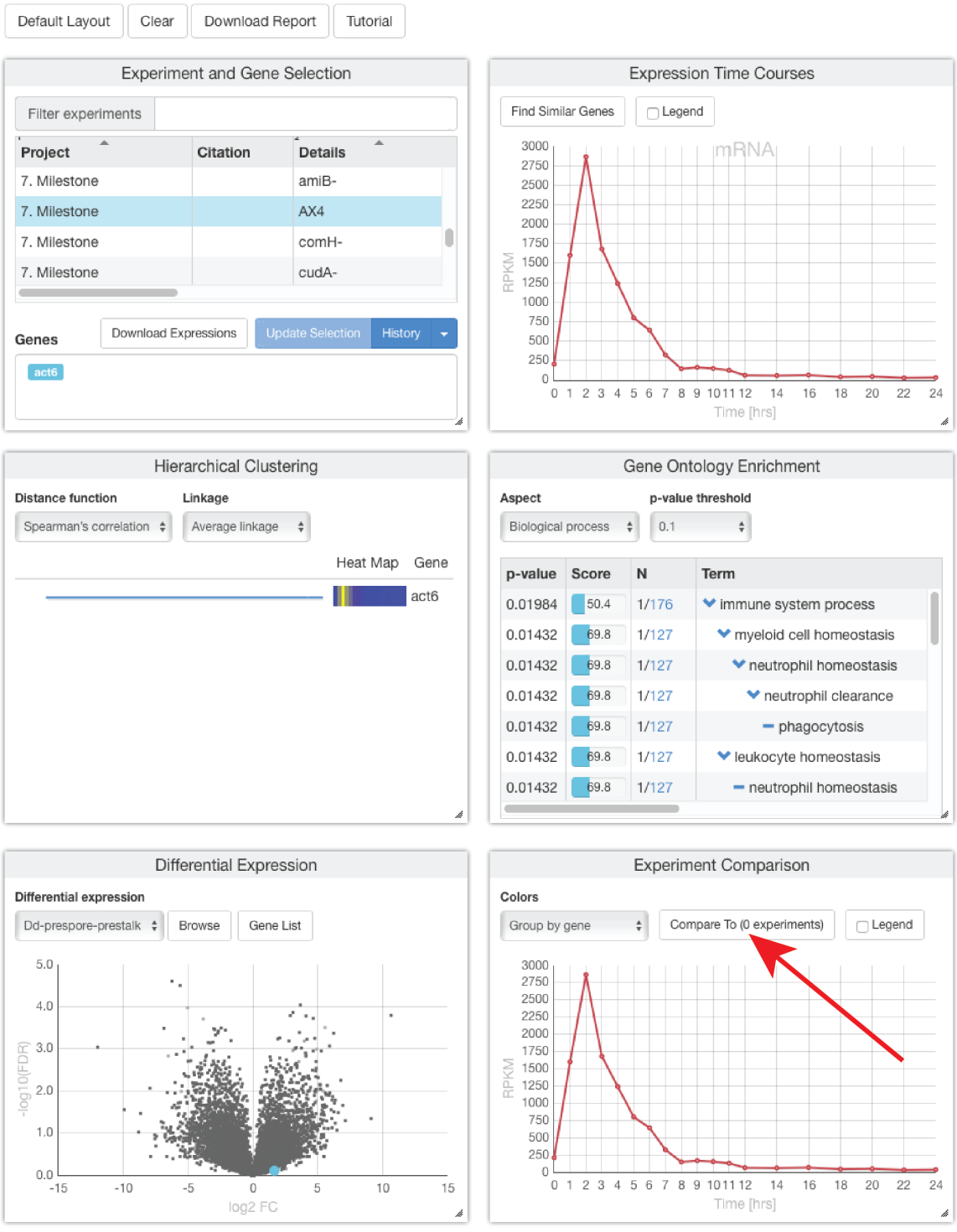
Type a gene name in the ‘Genes’ box. In this example we chose the actin gene Act6 by typing ‘act’ and selecting a gene from the ensuing drop-down menu.



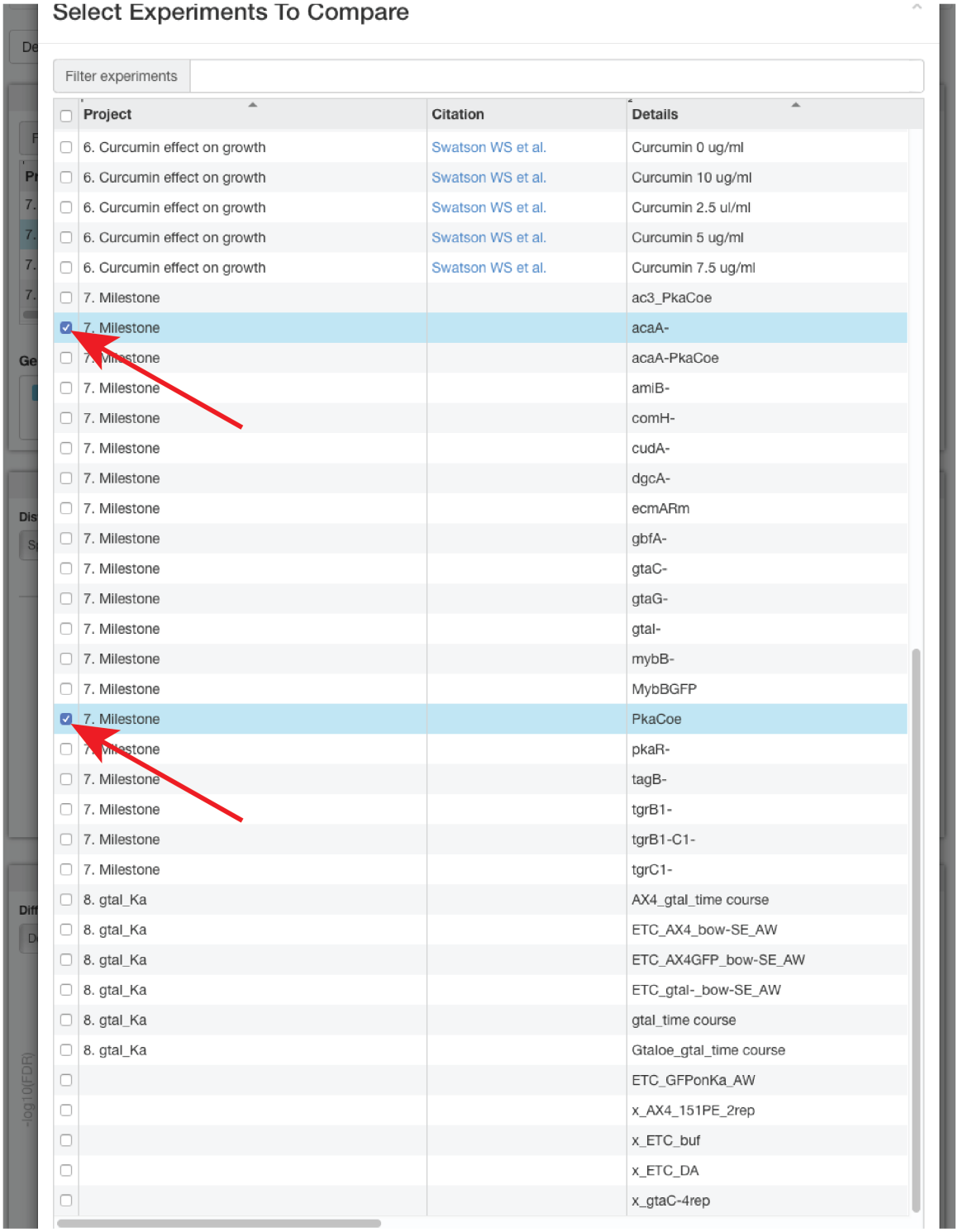
Pressing the ‘Update Selection’ button or hitting ‘return’ on your keyboard propagates the gene selection in all the other panels.



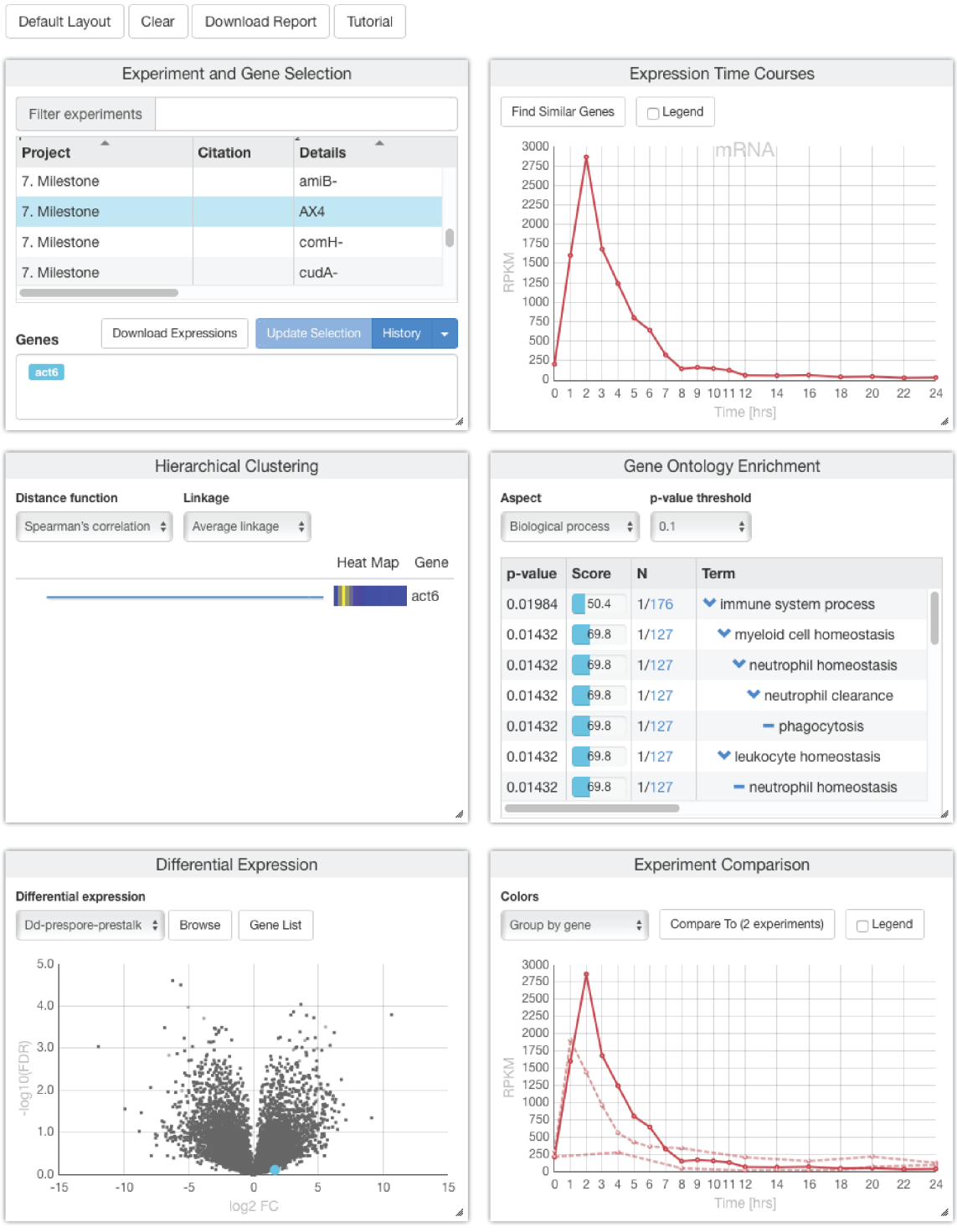
To compare the temporal and developmental expression patterns of actin 6 between several strains, click the ‘Compare To’ button in the ‘Experiment Comparison’ panel.



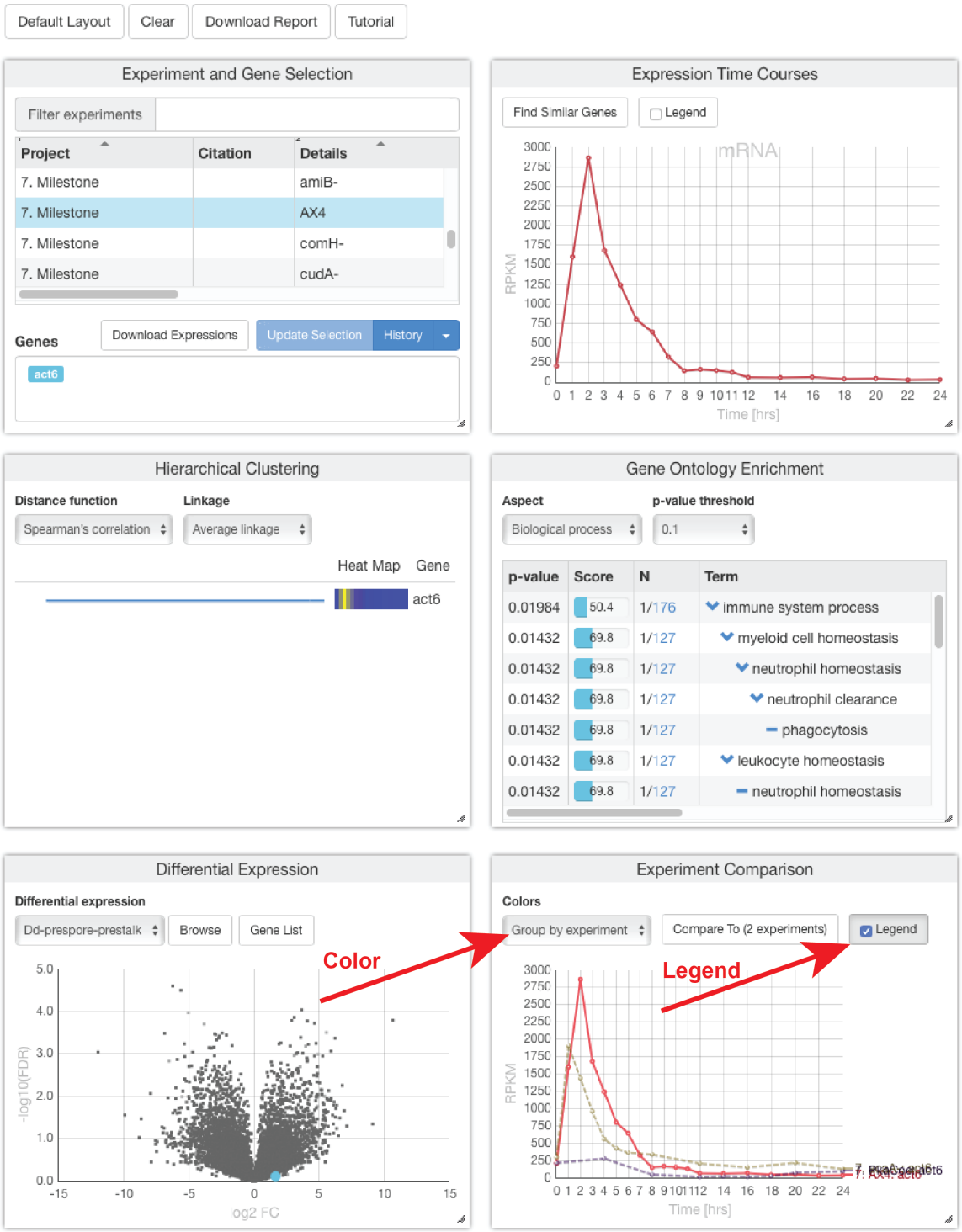
Select the desired experiments from the pop-up menu. Here, we selected the aggregationless mutant *acaA*$^{-}$ and the precocious developer *pkaC*^{OE}.



Close the selection menu to observe the comparison.



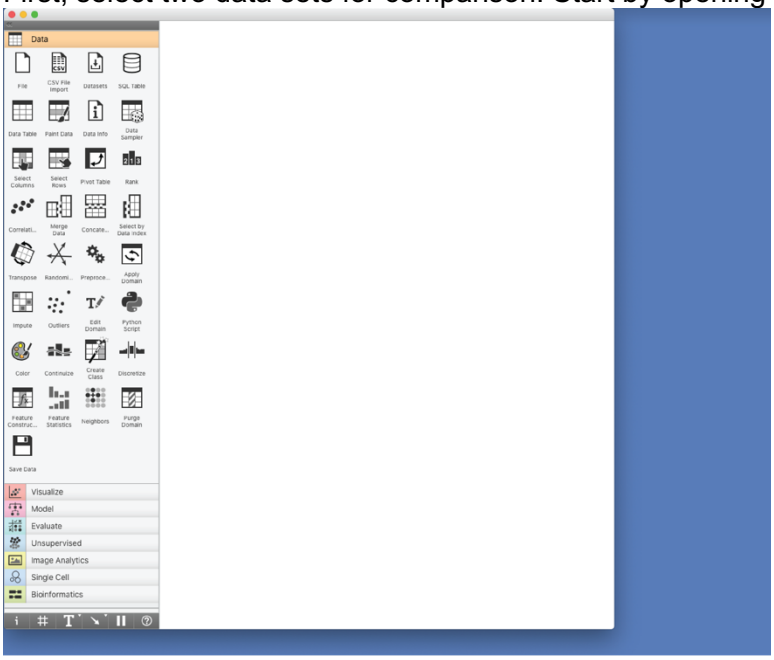
Change the color selection by selecting the desired grouping in the ‘Group by’ button. Add a legend as needed. Mouse over the legend to view details (not shown).
It is easy to see that the actin 6 mRNA abundance is reduced to about 60% in the aggregationless *acaA*$^{-}$ strain (tan) and to about 10% in the precocious *pkaC*^{OE} strain (purple).



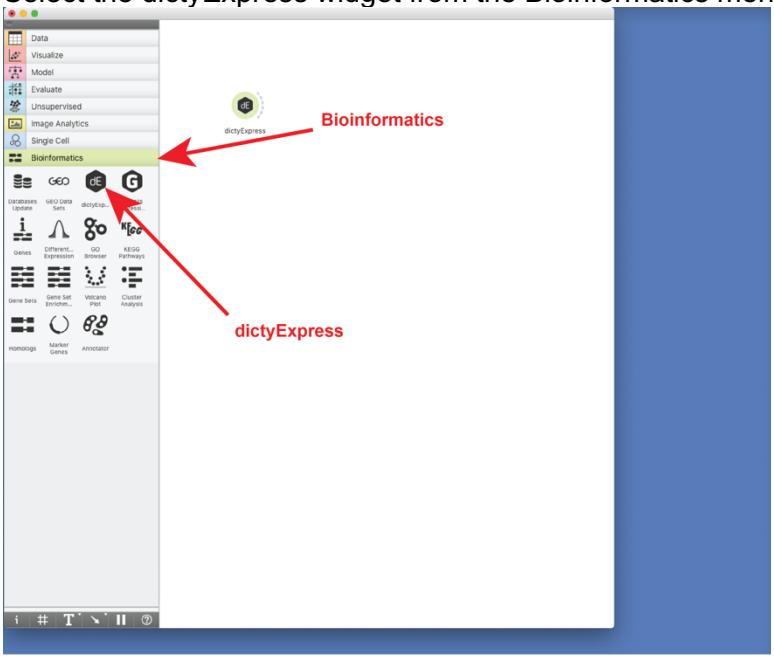
B. An introduction to Orange

Install Orange on your computer https://orangedatamining.com. If you are new to Orange, we strongly recommend following the 'Getting Started with Orange' tutorials at https://www.youtube.com/channel/UClKKWBe2SCAEyv7ZNGhIe4g
Then, add the Bioinformatics add-on as described in https://www.youtube.com/watch?v=OANsA6fMJKg. In the following introduction, we used Orange 3.27.1 with the Bioinformatics add-on 4.3.1

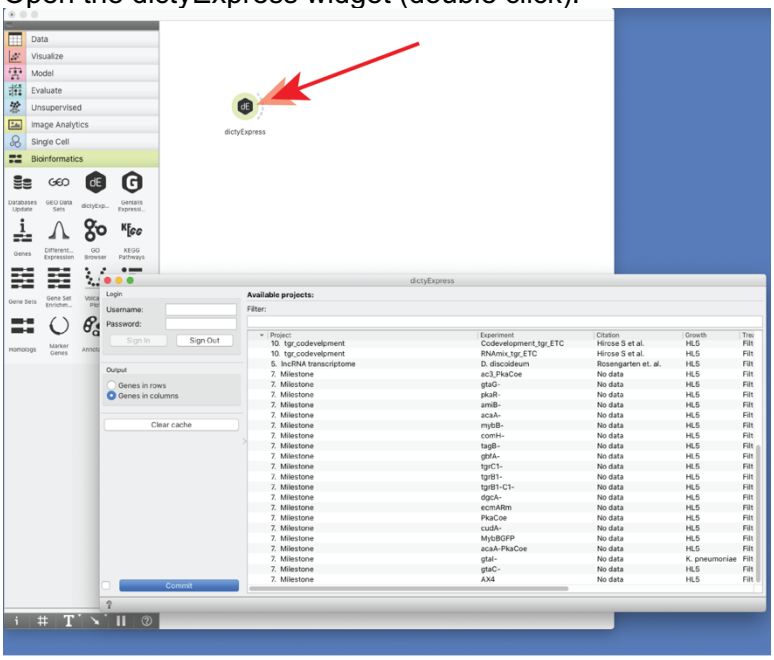
First, select two data sets for comparison. Start by opening a new Orange canvas.



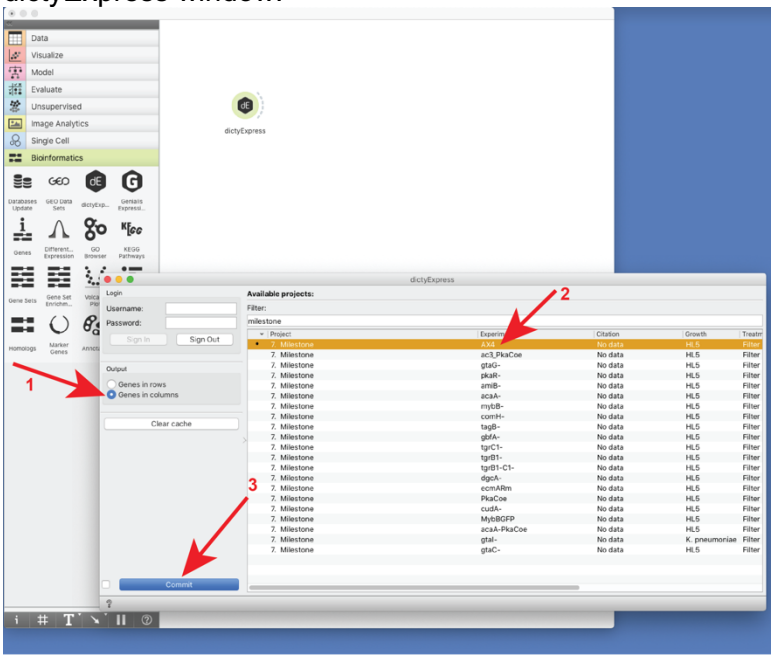
Select the dictyExpress widget from the Bioinformatics menu.



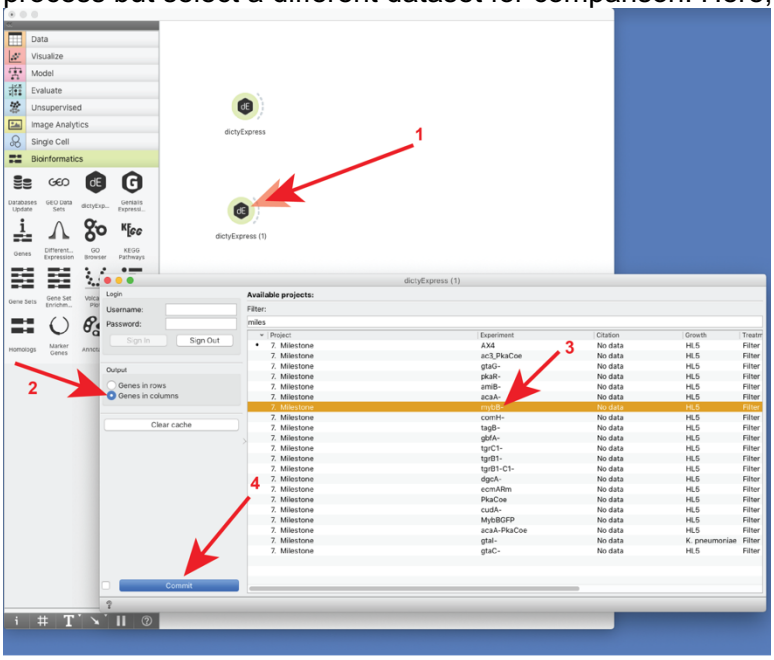
Open the dictyExpress widget (double click).



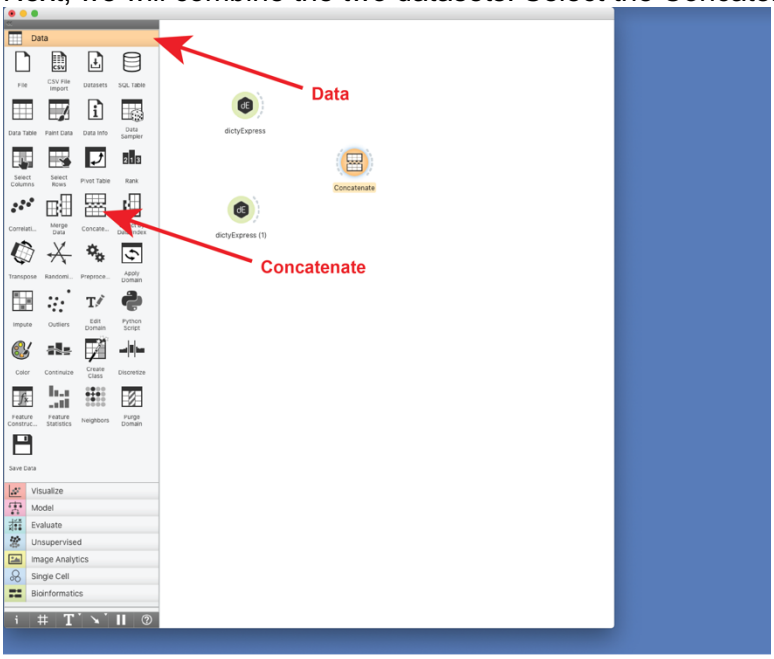
Select the ‘Genes in columns’ output and one of the Milestone datasets – a dot will appear next to the selection. Here, we selected AX4. Press ‘Commit’ to confirm your selection and close the dictyExpress window.



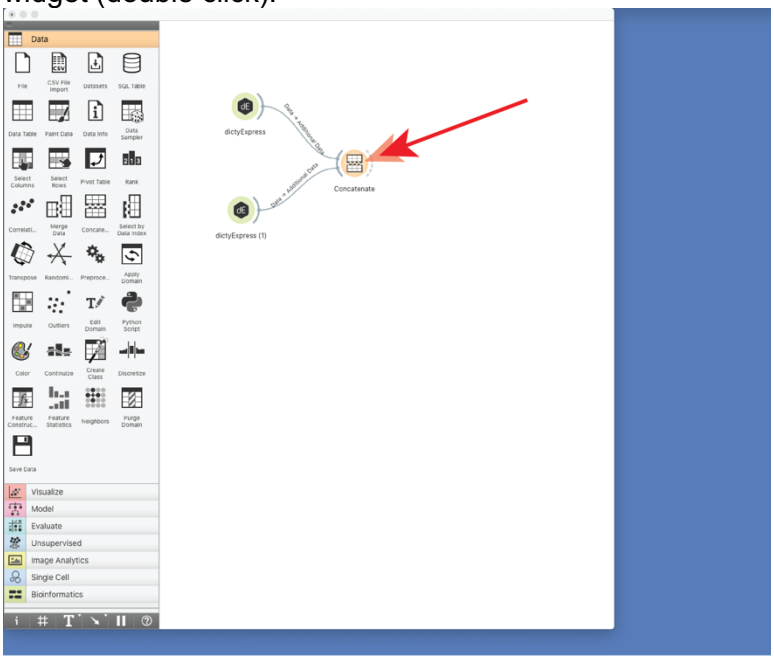
Select a second dictyExpress widget from the Bioinformatics menu and repeat the above process but select a different dataset for comparison. Here, we selected mybB-.



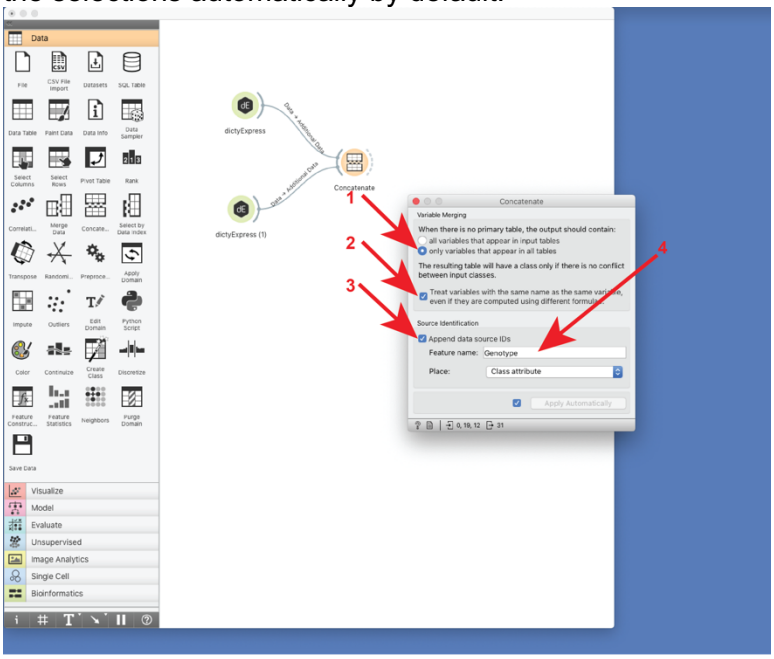
Next, we will combine the two datasets. Select the Concatenate widget from the Data menu.



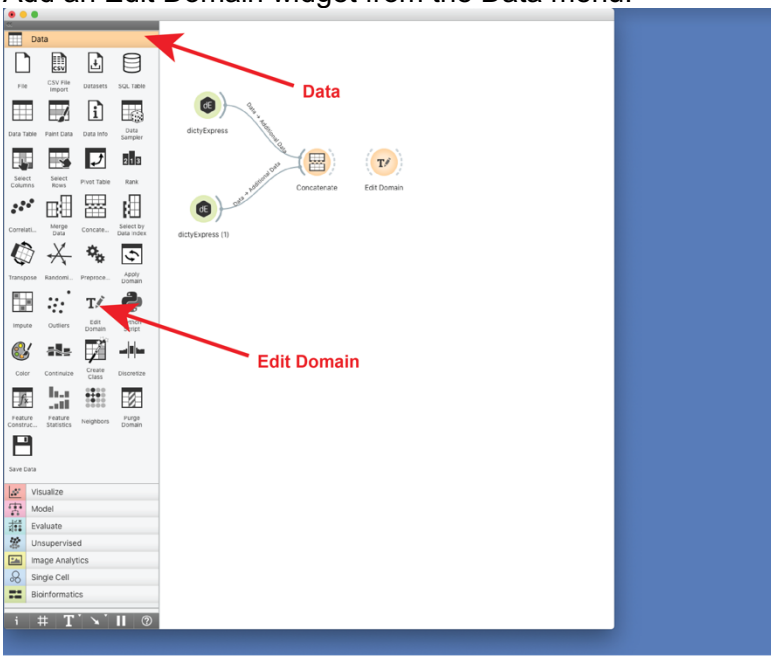
Connect the outputs of the two dictyExpress widgets to the Concatenate widget and open the widget (double click).



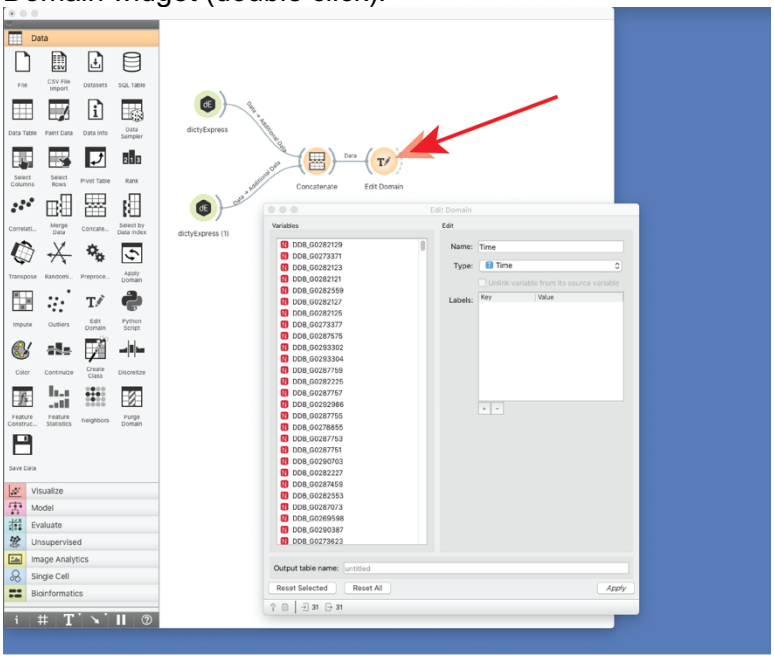
Select the variable merging and source identification options as shown. Here, we changed the source ID by typing ‘Genotype’ in the feature name box. Notice that this widget is set to apply the selections automatically by default.



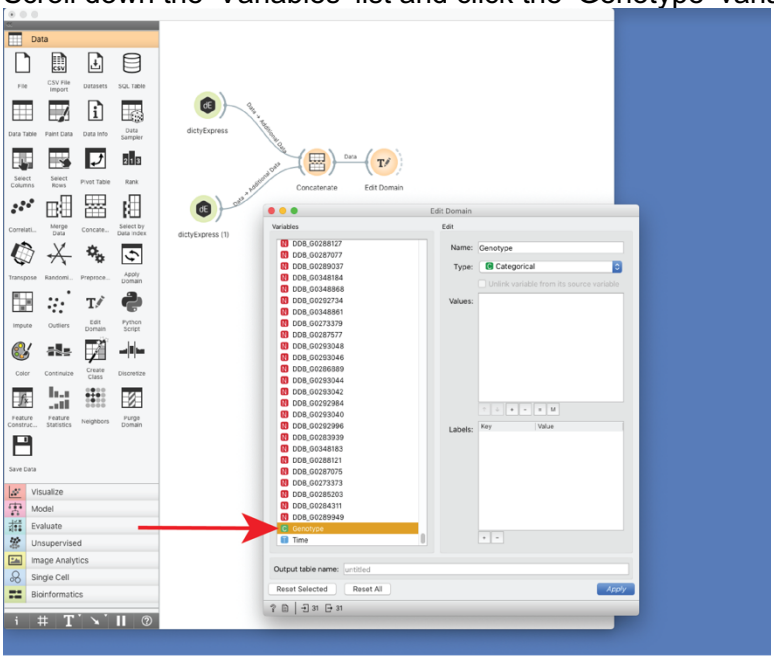
The next 5 steps will change the genotype labels to indicate the strain names in the output data.
Add an Edit Domain widget from the Data menu.



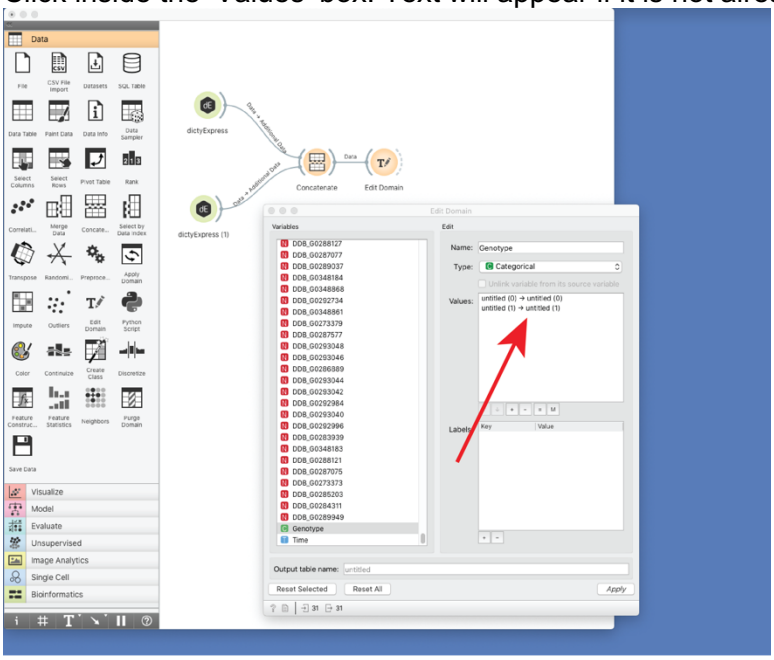
Connect the output of the Concatenate widget to the Edit Domain widget and open the Edit Domain widget (double click).



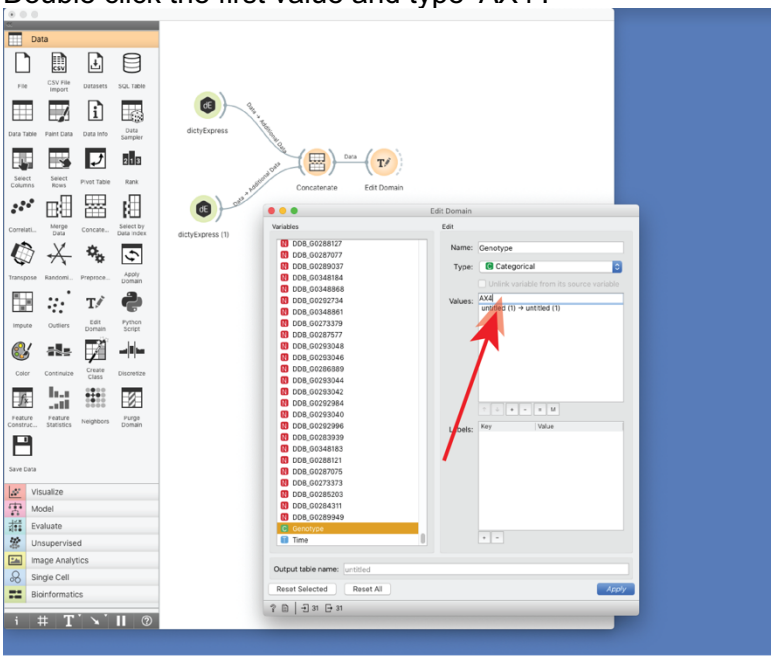
Scroll down the ‘Variables’ list and click the ‘Genotype’ variable.



Click inside the ‘Values’ box. Text will appear if it is not already there.

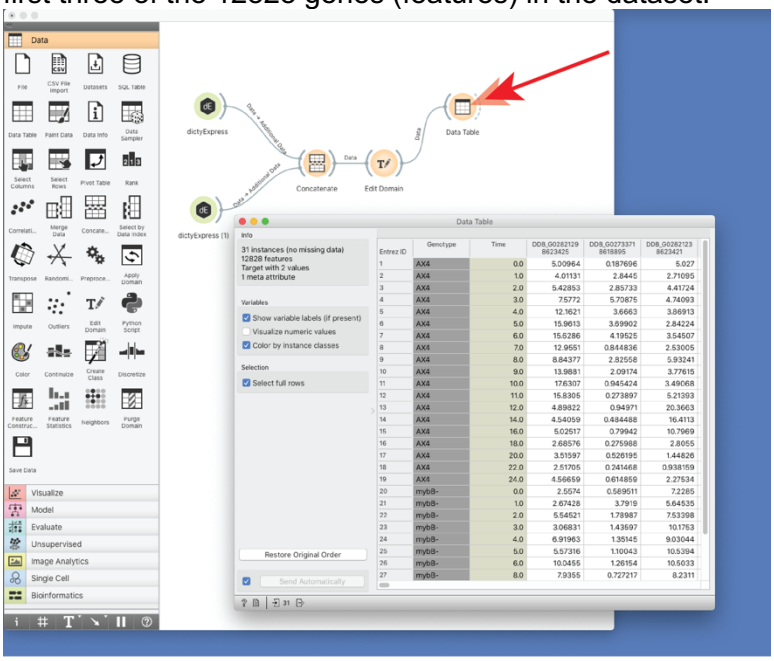


Double click the first value and type ‘AX4’.

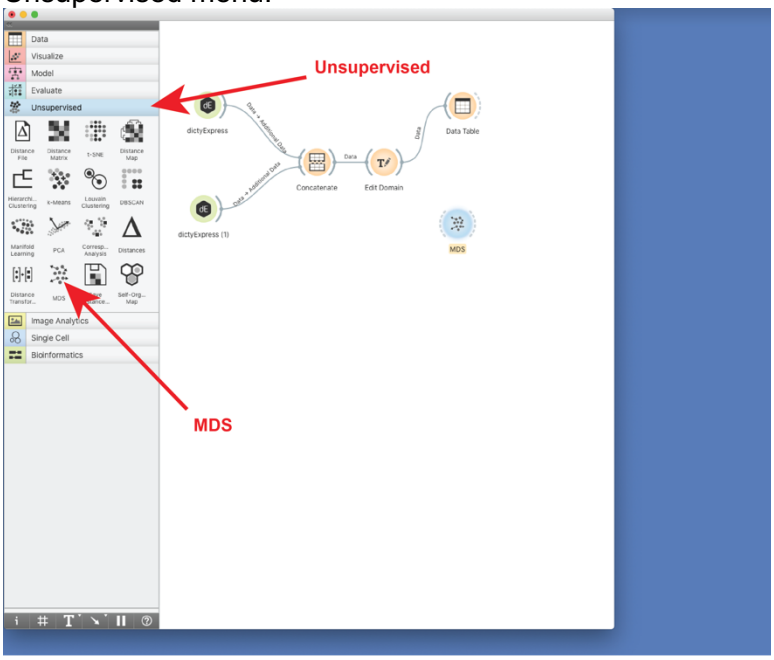


Double click the second value and type ‘mybB-‘. Press ‘Apply’ to propagate the two changes and close the Edit Domain window (not shown).

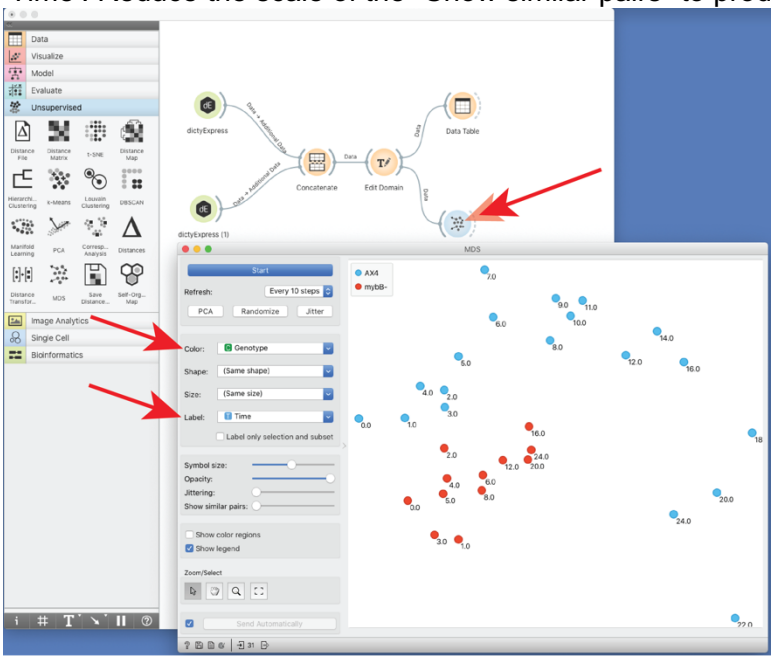
To view your combined data, connect a Data Table widget to the Edit Domain output and double click to view the data. Here we show the first few columns that list the Genotype, Time and the first three of the 12828 genes (features) in the dataset.



To compare the two datasets, select the MDS (multidimensional scaling) widget from the Unsupervised menu.



Connect the output of the Edit Domain widget to the MDS widget. Red dots will appear at the ends of the connecting line, indicating data processing. Open the MDS widget (double click). Using the interactive menu on the left, ‘Color’ the points by ‘Genotype’ and to ‘Label’ them by ‘Time’. Reduce the scale of the "Show similar pairs" to produce an MDS plot as shown.



The wild type (AX4) temporal progression is quite different from that of the aggregationless mutant (mybB-), similar to the data shown in Figure 2A in the main manuscript. The projection is not identical to Figure 2A because the latter contains additional datasets that affect the rendering. You could test your skills by changing the mutant dataset from mybB- to another mutant (suggestion: tagB-; not shown).

Supplemental_File_S5: Computational methods

Abbreviations:
lFC – log fold change
DE – differential expression/differentially expressed
padj – adjusted p-value
pval – p-value
RPKUM – Read counts Per Kilobase of exon model per Uniquely mapped Million reads
tt – transition time
agg – aggregation
FB – fruiting body
GAM – generalized additive model

1 Milestones

To find genes that change their expression relatively strongly between two consecutive developmental stages, we filtered the genes based on DE between two stages and also the shapes of their expression profiles. This approach ensured that the milestone gene exhibited a strong change between two stages and did not fluctuate much during the rest of development. For this analysis, we used AX4 samples annotated with developmental stages based on images that captured the morphologies of developmental structures. When an image contained multiple morphological stages, the majority morphology was used.

1.1 Genes with marked expression changes between stages

First, DE analysis was performed with the DESeq2 (v1.26.0) R library (Love et al. 2014) for every pair of neighboring stages using the AX4 samples. The later stage was used as the case and the earlier as the control. The padj was re-calculated over all the tests for all neighboring stage pairs using Benjamini-Hochberg correction on the pval from DESeq2. A gene was considered DE if the absolute lFC ≥ 2 and padj ≤ 0.01.

1.2 Genes differentially expressed across stages

The shape of the expression profile was analyzed with the ImpulseDE2 (v1.10.0) R library (Fischer et al. 2018) to find DE genes whose expression profiles change monotonously or transiently during development. The ImpulseDE2 model was fit to the AX4 data using ordered majority stages converted to consecutive integers as time-points. Transition times were defined as the x-coordinate values of the sigmoid midpoints. The analysis was run in case-only mode with identification of transient genes whose expression profiles are better fit by single or double sigmoid model compared to constant model. Genes were considered significantly DE between two stages based on padj threshold = 0.001 and with a tt between those stages.

The fitted ImpulseDE2 models were parsed to obtain neighboring stages that had tt between them, indicating a change in the expression level. The tt values were extracted from the appropriate model based on genes that were termed as monotonously or transiently changed across stages by ImpulseDE2. In some cases, this would lead to an inappropriate assignment of tt owing to the input timescale range (stages) and model complexity (single or double sigmoid). For each type of tt that would lead to an inappropriate expression change assignment (described below), changes were made to

the tt values based on visual evaluation of example genes with the same tt value inconsistency.
We extracted a single tt for monotonously DE genes according to the following steps even when the double sigmoid impulse model (i.e. having two tt values) was used. If the padj of the impulse model was lower than that of the sigmoid model (single sigmoid), the impulse tt closer to the tt of the sigmoid model was chosen. Otherwise, the tt of the sigmoid model was used. For border reassignment, if a tt value was smaller than the x value of the first stage (no agg), it was readjusted to be immediately after the first stage. If a tt was larger than the x-value of the last stage (FB), it was readjusted to be immediately before the last stage.
For transiently DE genes, two tt values were extracted from the double sigmoid model. If both tt values were between the same two stages, they were reassigned to be right before the first neighboring stage and right after the second neighboring stage. If tt values were smaller than the first stage (no agg) or larger than the last one (FB), they were reassigned as in the monotonous model. If this procedure set the two tt values to be between the same two stages, a single tt value between these two stages was extracted.

1.3 Selection of milestone genes

A gene was determined to be a milestone between two neighboring stages by two criteria; 1) the gene was significantly DE between these two stages based on DESeq2 (Section 1.1) and 2) it was significantly DE based on ImpulseDE2 with tt between the two stages (Section 1.2). These milestone genes were then separated based on being up- or down-regulated between the two stages.

1.4 Milestone genes - expression heatmaps

The expression profiles of milestone genes were visualized with the ComplexHeatmap (v2.3.3) R library (Gu et al. 2016). RPKUM data were averaged across the multiple samples, which were annotated as the same majority stage in each strain, so that the expression data of each gene were summarized by a single averaged value for each stage of a strain. The expression was scaled based on the following formula:

$$Gist\ scaled = \frac{Gist - p_{99}(Gi)}{p_{99}(Gi)}$$

where *Gi* represents all the averaged expression values of a given gene (*i*) across stages and strains, *Gist* represents *Gi* in a certain strain (*s*) at a certain stage (*t*), and p_{99} represents the 99th percentile. This formula linearly scales the majority of the *Gist* values to the interval [-1,0] with extremely high *Gist* being given a value above zero. Values were then capped at 0.1 to reduce the effect of extreme values on the color scale.
The heatmaps of the milestone genes were prepared separately for each pair of neighboring stages and for up- and down-regulation. Gene ordering was based on hierarchical clustering of the scaled averaged AX4 data with the Ward algorithm (ward.D2) in hclust (R v3.6.3) using Euclidean distances, followed by visual reordering with the seriation (v1.2-8) R library (Hahsler et al. 2008). The two strains that did not have stage annotations (*ac3^{-}/pkaCoe* and *gtaC^{-}*) were not included in the milestones heatmap.

2 Regulons

2.1 Selection of regulon candidate genes

Co-regulated gene pairs were extracted from individual strain data to avoid biasing in favor of strains with more samples, according to the following steps. First, we excluded genes whose RPKUM was all-zero in a strain. The RPKUM values of each gene were transformed by adding a pseudocount (+1) followed by $\log_2$ transformation and scaling to a mean = 0 and standard deviation = 1. We used the Python nearest neighbor descent, PyNNDescent (v0.3.3) library (https://libraries.io/pypi/pynndescent/0.3.3), to obtain the 300 nearest neighbors of each gene based on cosine similarity. Then, we chose all the genes that have at least one nearest neighbor that exhibited a similarity equal to or higher than the strain-specific threshold (the 30th percentile of the similarities in each strain; see 2.2 Strain-specific similarity threshold). For each gene, we counted the number of strains in which the gene was found to have some co-expressed neighbor(s) and compared the number with the gene-specific N threshold. The gene-specific N was specified by the number of strains in which the gene was deemed as expressed highly enough (see details below). If the gene had closest neighbor(s) present in at least N strains, it was considered a regulon candidate. Some genes were strongly co-regulated only in a few strains and exhibited mainly low or no expression in most strains, where they were not counted as co-regulated. When the expression level of a gene pair is very low, cosine similarity often becomes lower than the true similarity due to amplifying noise. Therefore, even if the genes are co-regulated, they would be counted as negative. To avoid such false negatives, we lowered the gene-specific N threshold by not taking into account strains in which the gene was expressed at very low levels. Thus, we first determined the H percent of the 99th percentile expression (*He*) in all samples and then defined the gene-specific N threshold as the number of strains in which the gene's expression reached its *He* value at any timepoint. We also tested different H values (0, 10, 30, or 50) for the expression level. By increasing H (resulting in lowering N) more genes would be included as regulon candidates, because genes can be co-expressed even in strains that exhibit relatively low expression (empirically, we found H=0, 365 genes; H=10, 1099 genes; H=30, 1974 genes; H=50, 3182 genes). We chose H=10 based on a visual inspection of the regulons obtained with different values of H. Moreover, we also set an upper limit of N=18 instead of 21 (all strains) to avoid more false negatives in the extraction process for the co-expressed gene pairs. The cap N=18 ensures that a gene would have co-expressed close neighbor(s) in at least one strain among the aggregation minus group in which regulons were frequently disrupted.

2.2 Strain-specific similarity threshold

A gene profile similarity threshold was selected to classify genes as co-expressed or not. The gene profile similarities depend on the strain-specific number of samples and data quality. A different similarity threshold was thus selected for each strain. These similarities to the top closest neighbors obtained for each strain displayed a left-skewed distribution. A strain-specific similarity threshold was set to the 30th percentile of the similarities to the closest neighbors. This threshold approximately separated the closest neighbor similarity distribution to a bulk of genes with close neighbors and a tail of genes that had relatively low similarity to the closest neighbor.

2.3 Clustering of selected genes into regulons

The selected regulon candidate genes were clustered based on their expression profiles. Clustering and data preprocessing were performed in Orange (v3.26) (Demsar et al. 2013). We used two methods for data preprocessing: 1) We added a pseudocount (+1) to the RPKUM data, $\log_2$-transformed and scaled to mean = 0 and standard deviation = 1. We used the scaled expression of samples as features for clustering. 2) We scaled the RPKUM data of each gene to interval [0,1], followed by PCA dimensionality reduction. We used the first 30 PCA components as features for clustering. Based on visual evaluation of the clustering results, we selected the first method for AX4-based regulons. Most of the regulon candidate genes are expressed in AX4 and thus the first method performed better on the AX4 data as it was able to capture more subtle changes in expression. On the other hand, the second method gives more importance to higher expression profiles, mostly due to peaks, and less importance to lower expression values than the first method. Thus, the second method performed better on strain-wide data where many strains do not express a gene leading to relatively more noise in their low expression values. We selected the second method for all-strains-based regulons. Louvain clustering was performed with resolution 0.8 when AX4-based regulons were extracted from the AX4 data only and with resolution 0.4 for all-strains-based regulon extraction.

2.4 Regulons expression heatmap

Regulon heatmap were prepared as for Milestone genes with the following changes. RPKUM data were averaged across timepoints of the replicates of each strain. Regulons were ordered based on the developmental time of the gene expression peak in AX4. Peak times of individual regulon genes were obtained from the averaged non-scaled AX4 data. Regulons were ordered first by the median of the peak times, followed by the mean of the peak times of the regulon genes. Genes were ordered within each AX4 regulon separately. The ordering was based on hierarchical clustering of the scaled averaged AX4 data as for the milestone genes. The genes in the heatmap of the 'all-strains' regulons were ordered based on the AX4 order, first by regulons and then by ordering within the regulons.

3 Disaggregation genes

We performed DEseq2 analysis to select genes that are related to the disaggregation process in the *tgr* mutant strains. The relevant timepoints were selected based on visual evaluation of the differences in PC1 values between *tgrB1*$^-$ and *tgrB1*$^-$*C1*$^-$ and the AX4, *tagB*$^-$ and *comH*$^-$ strains.

3.1 Selection of disaggregation genes

Genes that were upregulated during disaggregation, but not upregulated at the same time in normal development were extracted with the following method. Genes DE in individual strains between time points: 6 and 8 hrs, and 8 and 12 hrs, were extracted for AX4, *tagB*, *comH*, *tgrB1*$^-$, and *tgrB1*$^-$*C1*$^-$ strains with DESeq2. The design used adjustment for replicates and thus only replicates present at both timepoints were used. The DESeq2 results were optimized for padj threshold 0.01. A gene was considered significantly upregulated if lFC ≥ 1.32 and padj ≤ 0.01. For each time comparison, the genes upregulated during disaggregation in both *tgrB1*$^-$ and *tgrB1*$^-$*C1*$^-$, but not in AX4, *tagB*$^-$, or *comH*$^-$ were selected as disaggregation genes.

3.2 Comparison between the disaggregation and dedifferentiation genes

To characterize the selected disaggregation genes, they were compared with genes that are upregulated during early dedifferentiation. The dedifferentiation genes were obtained from published dedifferentiation RNA-seq data (Nichols et al. 2020). The published data included an experiment in which cells were disaggregated and incubated in nutrient medium to induce dedifferentiation, and a control in which the disaggregated cells were incubated in non-nutrient buffer. We downloaded the RNA-seq fastq files (GSE144892) and prepared the RPKUM data by the same procedure as ours through the Genialis platform. Dedifferentiation genes were selected based on a DESeq2 comparison between the pooled 'medium' samples at 0.5, 1, and 2 hrs and the pooled 'buffer' samples at 0, 0.5, 1, 2, 3, 4, and 6 hrs. We tested for upregulation with DeSeq2 and optimized the results for padj threshold 0.01. A gene was considered to be upregulated during dedifferentiation if IFC ≥ 2 and padj ≤ 0.01. Gene expression scaling and gene ordering for the heatmaps were performed as for the milestone genes. We used a hypergeometric test to determine whether the disaggregation genes significantly overlap with the dedifferentiation genes. For the reference group in the test we used all genes expressed in the data published with this paper.

4 Developmental stage annotation

We prepared two types of stage annotations: 1) all stages annotations and 2) representative stages annotations. First, we manually annotated developmental stages from the microscopic images showing developmental morphological structures. When an image contained multiple morphological stages, we annotated the sample with all the observed structures. If the image was not captured for any sample, it was annotated as "no image", except for t=0 where it was annotated as no_agg. All stage annotations are shown in the color pallet above the heatmaps of regulons and disaggregation genes as information for each sample. When selecting milestone genes that transcriptionally define each developmental stage boundary, we annotated each sample with a "representative stage annotation" that is characteristic of the most abundant morphology.

5 Gene-set enrichment analysis

Datasets used for gene-set enrichment (including dictyBase gene name – entrez ID mapping and gene sets) were obtained on the 5th of April, 2020. The data were collected from the following sources. Gene information and taxonomy data were obtained from the NCBI database (ftp://ftp.ncbi.nlm.nih.gov). Gene ontology and KEGG pathway information was obtained from the official GO (http://geneontology.org/docs/download-ontology/) and KEGG (https://www.genome.jp/kegg/) knowledge-bases, respectively. The gene sets were pre-processed to contain only genes with Entrez IDs. The code used for data retrieval is available at https://github.com/JakaKokosar/bioinformatics-serverfiles and the data used in this study are stored at http://download.biolab.si/datasets/bioinformatics/2020_04_05/.

The analysis was performed using the Orange Bioinformatics (v4.0.0) Python library. Entrez IDs were mapped to the dictyBase gene names. The following gene sets were used: generic GO slims for biological process, molecular function, and cellular

component; KEGG Pathways; and the custom gene sets described below. Only gene sets with size within the interval [5,500] were used. The size was determined based on the number of genes that were contained within the reference set. The reference set contained all the genes present in the RNA-seq data, except those that had all zero expression values. Reference and query sets were filtered to include only genes that had an Entrez ID and were contained within at least one of the used gene sets. The latter was done to account for different proportions of genes annotated with a gene set between the reference and the query groups. All gene sets that had non-zero overlap with the query were tested for enrichment using the Orange Gene Set set_enrichment function. The pval were adjusted with Benjamini-Hochberg correction. Results were filtered to display only gene sets with padj ≤ 0.25 and overlap with query ≥ 2.

5.1 Custom gene sets

We used cell-type specific genes (Prespore and Prestalk genes) (Parikh et al. 2010), cAMP-pulse induced genes (Iranfar et al. 2003), chemotaxis genes (Swaney et al. 2010), *Dictyostelium* short gene families (*hssA/2C/7E* family, 57-aa protein family, *sig* and sigN genes, and *gtaG*-dependent short proteins) (Shimada et al. 2008; Vicente et al. 2008; Katoh-Kurasawa et al. 2016) and transcriptional regulation and chromatin organization (regulatory transcription factor, general transcription factors, mediators, chromatin remodeling/histone modification, histone/histone variants, and chromatin/centromere) (Rosengarten et al. 2013; Forbes et al. 2019). These custom gene sets are provided in Supplemental_File_S8.

Supplemental_File_S6: Standard experimental methods

Cell culture, strain maintenance, development and spore collection

All the *Dictyostelium discoideum* strains were derivatives of AX4 (Knecht et al. 1986) as detailed in Supplemental_Table_S1. We cultured cells at 22°C in HL5 medium with the necessary supplements and antibiotics. To induce development, we washed exponentially growing cells twice with KK2 buffer (20 mM potassium phosphate, pH6.4) to remove nutrients. Cells of the *gtaI*$^{-}$ strain were grown in association with live *Klebsiella pneumoniae* bacteria on SM plates, collected at the exponential growth phase, and washed at least three times with DDW. In all cases, we deposited the cells at a density of $2.6x10^6$ cells/cm^2 on black nitrocellulose filters on top of a paper pad soaked with PDF buffer (20 mM KCl, 9.2 mM K_2HPO_4, 13.2 mM KH_2PO_4, 5.3 mM $MgCl_2$ and 1 mM $CaCl_2$, pH6.4). The cells were incubated in the dark at 22°C for defined periods of time (Katoh et al. 2004). We collected spores from developing structures and treated with detergent (0.1% NP-40, 1mM EDTA in KK2 buffer) to eliminate amoebae (Shaulsky and Loomis 1993).

RNA-seq

We collected the cells from one nitrocellulose filter at each time point of two to seven independent developmental series, extracted total RNA from each sample using 1 ml of Trizol (Invitrogen) and performed poly(A) selection twice as described (Katoh-Kurasawa et al. 2016). We note that fruiting bodies, which contain walled spores and stalk cells, were not broken mechanically prior to RNA extraction, which could have resulted in underrepresentation of RNA species that are found exclusively in these walled cells (Van Driessche et al. 2005). We prepared multiplexed cDNA libraries and performed RNA sequencing using the Illumina sequencing platform as described previously. We mapped the resulting sequences to the *Dictyostelium* reference genome and obtained mRNA abundance values for each gene in the genome (Miranda et al. 2013) through the web applications dictyExpress, GenBoard or Genialis platform. The data were deposited in GEO (accession numbers GSE152851). Unless otherwise stated, we preprocessed RPKUM data by log_2-transformation after adding one (pseudocount), and then scaling to mean = 0 and standard deviation = 1.

Supplemental Files S7 and S8 are provided as separate Excel spreadsheets.

Supplemental_File_S7 Milestones, regulons and disaggregation gene lists
Supplemental_File_S8 Reference gene lists

Supplemental_Table_S1 *D. discoideum* strains used

Strain name	Phenotype group	Strain Descriptor	Strain summary	Parental strain	Antibiotic resistance	Reference
AX4	wild type	AX4	wild type	AX3		(Knecht et al. 1986)
MybBGFP	wild type	mybB–/[mybB]:mybB:GFP	expressing MybB:GFP under control of *mybB* promoter in *mybB*–	*mybB*-	Blasticidin S, G418	this study
pkaC^{oe}	precocious development	[act15]:pkaC:HA	expressing PkaC under control of *act15* promoter in AX4	AX4	G418	this study
pkaR–	precocious development	*pkaR*–	*pkaR* null mutant	AX4	Blasticidin S	(Shaulsky et al. 1998)
acaA–/ *pkaC*^{oe}	small fruiting body	*acaA*– /[act15]:pkaC	expressing PkaC under control of *act15* promoter in *acaA*–	*acaA*-	Blasticidin S, G418	this study
ac3–/ *pkaC*^{oe}	small fruiting body	*acaA*–/*acgA*– /*acr A*–/ [act15]:pkaC:HA	expressing PkaC under control of *act15* promoter in *acaA*–/*acgA*– /*acrA*–	*acaA*- /*acgA*- /*acrA*-	Blasticidin S, G418	this study
gtaI–	culmination defective	*gtaI*–	*gtaI* insertional mutant	AX4	Blasticidin S	this study
gtaG–	culmination defective	*gtaG*–	*gtaG* insertional mutant	AX4	Blasticidin S	(Katoh-Kurasawa et al. 2016)
cudA–	culmination defective	*cudA*–	*cudA* deletion mutant pcudAKO(MF2)	AX4	Blasticidin S	this study; Plasmid: (Fukuzawa et al. 1997)
dgcA–	culmination defective	*dgcA*–	*dgcA* null mutant pdgcA_KO_443	AX4	Blasticidin S	this study; Plasmid: (Chen and Schaap 2012)
ecmARm	culmination defective	[ecmA]:pkaR (G135E/G261A)	*gtaG* insertional mutant	AX4	G418	this study
tagB–	tag arrest	*tagB*–	*tagB* null mutant	AX4	Blasticidin S	(Khare and Shaulsky 2010)
t345	tag arrest	*comH*–	*comH* insertional REMI mutant	AX4	Blasticidin S	(Kibler et al. 2003)[g]
tgrB1–	tag disaggregation	*tgrB1*–	*tgrB1* null mutant	AX4	Hygromycin B	(Benabentos et al. 2009)

tgrB1C1⁻	tag disaggregation	*tgrB1*⁻/*tgrC1*⁻	*tgrB1C1* null mutant	pyr5-6-	Uracil	(Hirose et al. 2011)
tgrC1⁻	lag disaggregation	*tgrC1*⁻	*tgrC1* null mutant	AX4	Hygromycin B	(Benabentos et al. 2009)
gbfA⁻	lag disaggregation	*gbfA*⁻	*gbfA* null mutant	AX4	BS-	this study
gtaC⁻	aggregation minus	*gtaC*⁻	*gtaC* deletion mutant	AX4	Blasticidin S	(Keller and Thompson 2008)
acaA⁻	aggregation minus	*acaA*⁻	*acaA* null mutant	AX4	Blasticidin S	(Stepanovic et al. 2005)
mybB⁻	aggregation minus	*mybB*⁻	*mybB* insertional mutant	AX4	Blasticidin S	this study
amiB⁻	aggregation minus	*amiB*⁻	*amiB* insertional mutant p82ClaI_amiB-REMI	AX4	Blasticidin S	this study; Plasmid: (Kon et al. 2000)

References

Benabentos R, Hirose S, Sucgang R, Curk T, Katoh M, Ostrowski EA, Strassmann JE, Queller DC, Zupan B, Shaulsky G et al. 2009. Polymorphic members of the lag gene family mediate kin discrimination in Dictyostelium. *Current biology : CB* **19**: 567-572.

Chen ZH, Schaap P. 2012. The prokaryote messenger c-di-GMP triggers stalk cell differentiation in Dictyostelium. *Nature* **488**: 680-683.

Demsar J, Curk T, Erjavec A, Gorup C, Hocevar T, Milutinovic M, Mozina M, Polajnar M, Toplak M, Staric A et al. 2013. Orange: Data Mining Toolbox in Python. *Journal of Machine Learning Research* **14**: 2349-2353.

Fischer DS, Theis FJ, Yosef N. 2018. Impulse model-based differential expression analysis of time course sequencing data. *Nucleic Acids Res* **46**: e119.

Forbes G, Chen ZH, Kin K, Lawal HM, Schilde C, Yamada Y, Schaap P. 2019. Phylogeny-wide conservation and change in developmental expression, cell-type specificity and functional domains of the transcriptional regulators of social amoebas. *BMC genomics* **20**: 890.

Fukuzawa M, Hopper N, Williams J. 1997. cudA: A Dictyostelium gene with pleiotropic effects on cellular differentiation and slug behaviour. *Development* **124**: 2719-2728.

Gu Z, Eils R, Schlesner M. 2016. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics* **32**: 2847-2849.

Hahsler M, Kurt H, Christian B. 2008. Getting Things in Order: An Introduction to the R Package seriation. *Journal of Statistical Software* **25**: 1-34.

Hirose S, Benabentos R, Ho HI, Kuspa A, Shaulsky G. 2011. Self-recognition in social amoebae is mediated by allelic pairs of tiger genes. *Science* **333**: 467-470.

Iranfar N, Fuller D, Loomis WF. 2003. Genome-wide expression analyses of gene regulation during early development of Dictyostelium discoideum. *Euk Cell* **2**: 664-670.

Katoh M, Shaw C, Xu Q, Van Driessche N, Morio T, Kuwayama H, Obara S, Urushihara H, Tanaka Y, Shaulsky G. 2004. An orderly retreat: Dedifferentiation is a regulated process. *Proceedings of the National Academy of Sciences of the United States of America* **101**: 7005-7010.

Katoh-Kurasawa M, Santhanam B, Shaulsky G. 2016. The GATA transcription factor gene gtaG is required for terminal differentiation in Dictyostelium. *J Cell Sci* **129**: 1722-1733.

Keller T, Thompson CR. 2008. Cell type specificity of a diffusible inducer is determined by a GATA family transcription factor. *Development* **135**: 1635-1645.

Khare A, Shaulsky G. 2010. Cheating by exploitation of developmental prestalk patterning in Dictyostelium discoideum. *PLoS genetics* **6**: e1000854.

Kibler K, Nguyen TL, Svetz J, van Driessche N, Ibarra M, Thompson C, Shaw C, Shaulsky G. 2003. A novel developmental mechanism in Dictyostelium revealed in a screen for communication mutants. *Dev Biol* **259**: 193-208.

Knecht DA, Cohen SM, Loomis WF, Lodish HF. 1986. Developmental regulation of Dictyostelium discoideum actin gene fusions carried on low-copy and high-copy transformation vectors. *Mol Cell Biol* **6**: 3973-3983.

Kon T, Adachi H, Sutoh K. 2000. amiB, a novel gene required for the growth/differentiation transition in Dictyostelium. *Genes to Cells* **5**: 43-55.

Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology* **15**: 550.

Miranda ER, Rot G, Toplak M, Santhanam B, Curk T, Shaulsky G, Zupan B. 2013. Transcriptional profiling of Dictyostelium with RNA sequencing. *Methods in molecular biology* **983**: 139-171.

Nichols JM, Antolovic V, Reich JD, Brameyer S, Paschke P, Chubb JR. 2020. Cell and molecular transitions during efficient dedifferentiation. *Elife* **9**.

Parikh A, Miranda ER, Katoh-Kurasawa M, Fuller D, Rot G, Zagar L, Curk T, Sucgang R, Chen R, Zupan B et al. 2010. Conserved developmental transcriptomes in evolutionarily divergent species. *Genome biology* **11**: R35.

Rosengarten RD, Santhanam B, Katoh-Kurasawa M. 2013. Transcriptional Regulators: Dynamic Drivers of Multicellular Formation, Cell Differentiation and Development. In *Dictyostelids: evolution, genomics and cell biology*, (ed. M Romeralo, et al.), pp. 89-108. Springer, Heidelberg ; New York.

Shaulsky G, Fuller D, Loomis WF. 1998. A cAMP-phosphodiesterase controls PKA-dependent differentiation. *Development* **125**: 691-699.

Shaulsky G, Loomis WF. 1993. Cell type regulation in response to expression of ricin A in Dictyostelium. *Developmental biology* **160**: 85-98.

Shimada N, Kanno-Tanabe N, Minemura K, Kawata T. 2008. GBF-dependent family genes morphologically suppress the partially active Dictyostelium STATa strain. *Devel Genes Evol* **218**: 55-68.

Stepanovic V, Wessels D, Daniels K, Loomis WF, Soll DR. 2005. Intracellular role of adenylyl cyclase in regulation of lateral pseudopod formation during Dictyostelium chemotaxis. *Euk Cell* **4**: 775-786.

Swaney KF, Huang CH, Devreotes PN. 2010. Eukaryotic chemotaxis: a network of signaling pathways controls motility, directional sensing, and polarity. *Annual review of biophysics* **39**: 265-289.

Van Driessche N, Demsar J, Booth EO, Hill P, Juvan P, Zupan B, Kuspa A, Shaulsky G. 2005. Epistasis analysis with global transcriptional phenotypes. *Nature genetics* **37**: 471-477.

Vicente JJ, Galardi-Castilla M, Escalante R, Sastre L. 2008. Structural and functional studies of a family of Dictyostelium discoideum developmentally regulated, prestalk genes coding for small proteins. *BMC Microbiol* **8:1**: 16 pages.