Supplementary Information for

Worm Perturb-Seq: massively parallel whole-animal RNAi and RNA-seq

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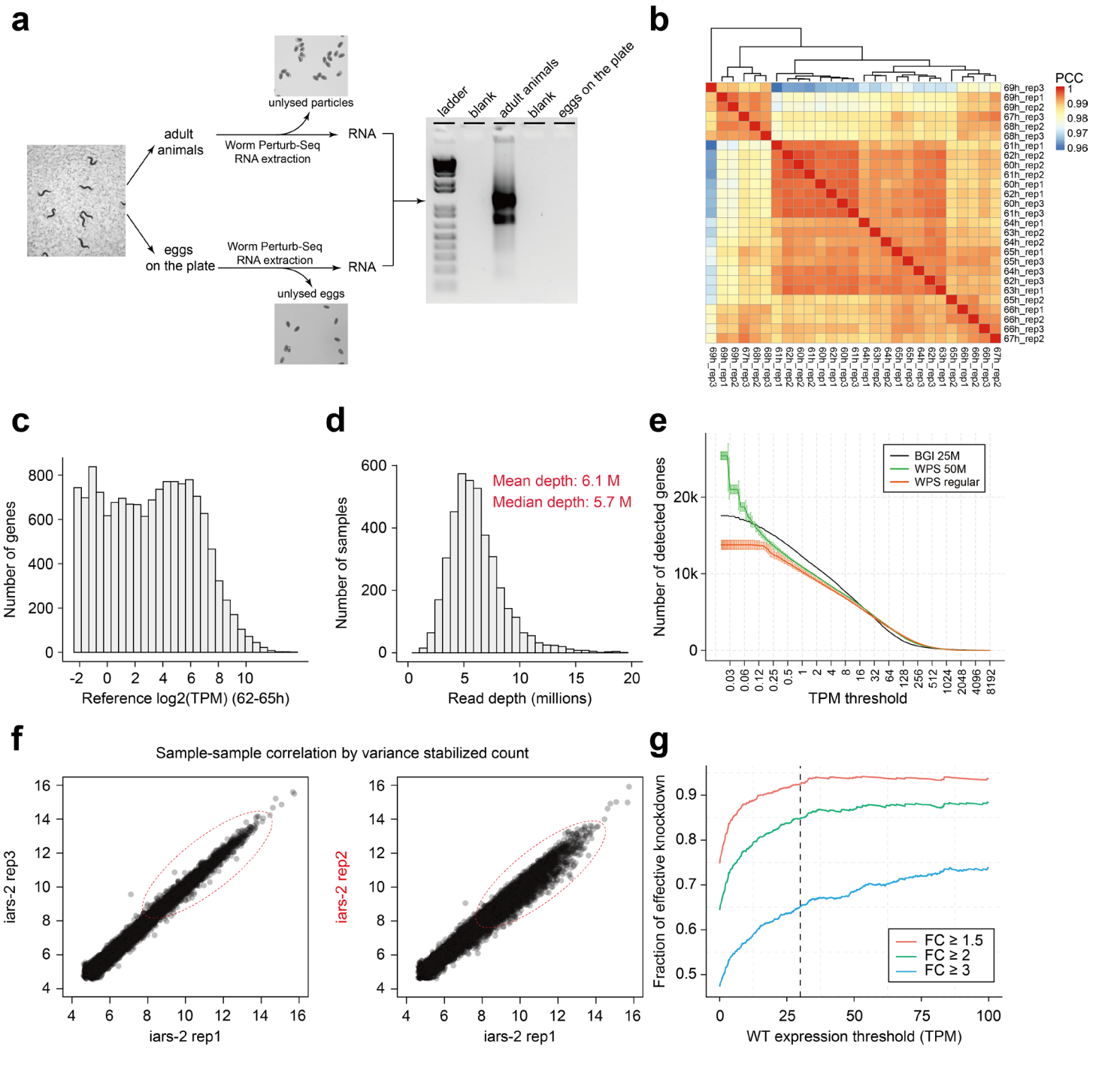
Supplementary Figures
Supplementary Notes
Supplementary Protocols

Other Supplementary Information for this manuscript include the following:

Supplementary Data 1: The WPS barcoding primers.
Supplementary Data 2: The TPM profile of the subsampling profiles used in this study. This is obtained from the combined vector control data collected at 62 to 65 hours post L1 stage for each biological replicate (See Supplementary Methods for details). The first replicate is used as reference profile.
Supplementary Data 3: List of 67 non-targeting perturbations (NTPs). The list does not include the 4 random spike-in vector controls.
Supplementary Data 4: Differentially expressed genes in NHR perturbations.
Supplementary Data 5: Perturbation-perturbation similarity matrix of NHR perturbations (only includes responsive conditions).

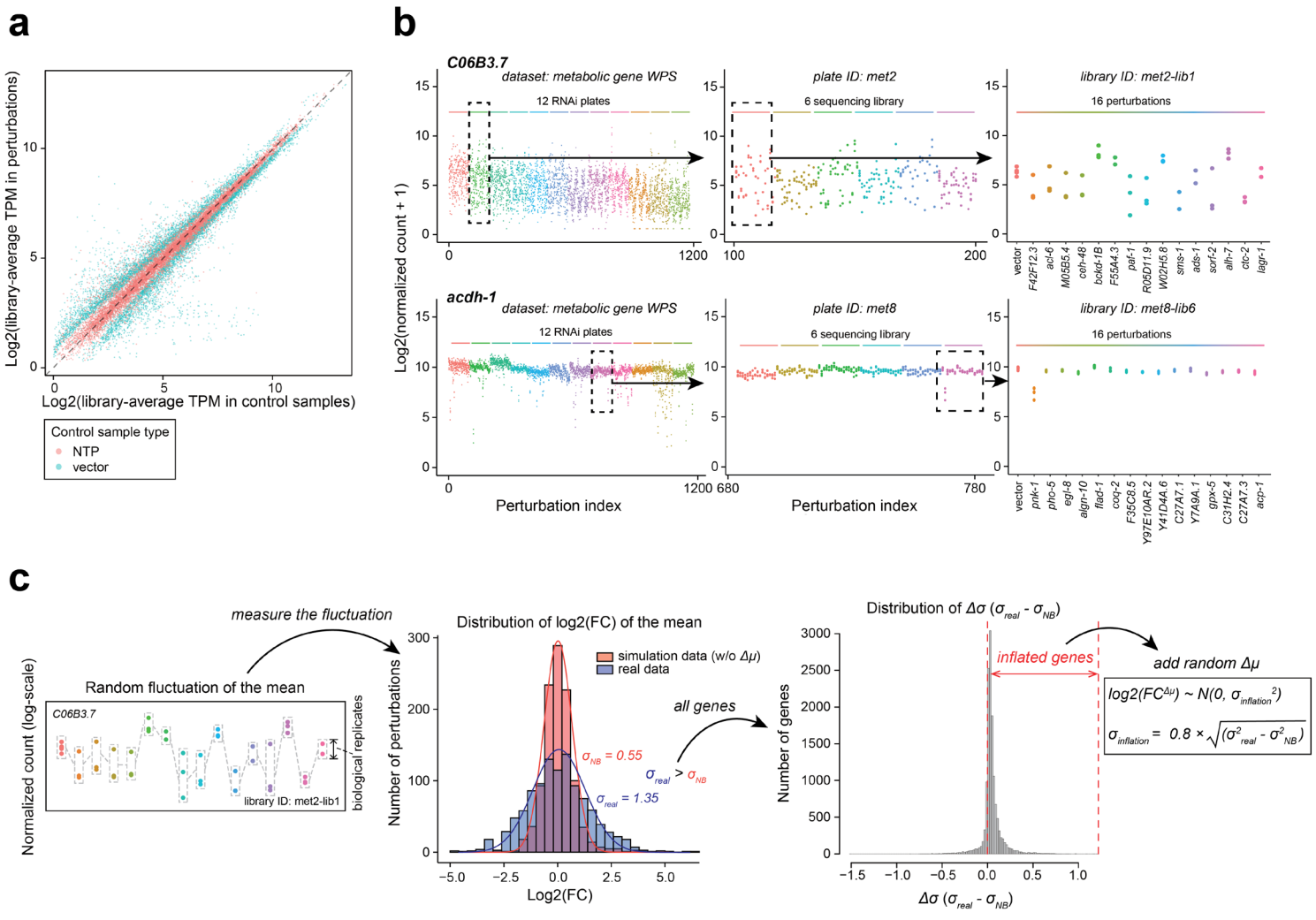


h

Intended target	Actual target
tre-5	*polg-1*
glod-4	*ipgm-1*
gsy-1	NTP (~130 bp dsRNA with 22 bp targeting to *gsy-1*)

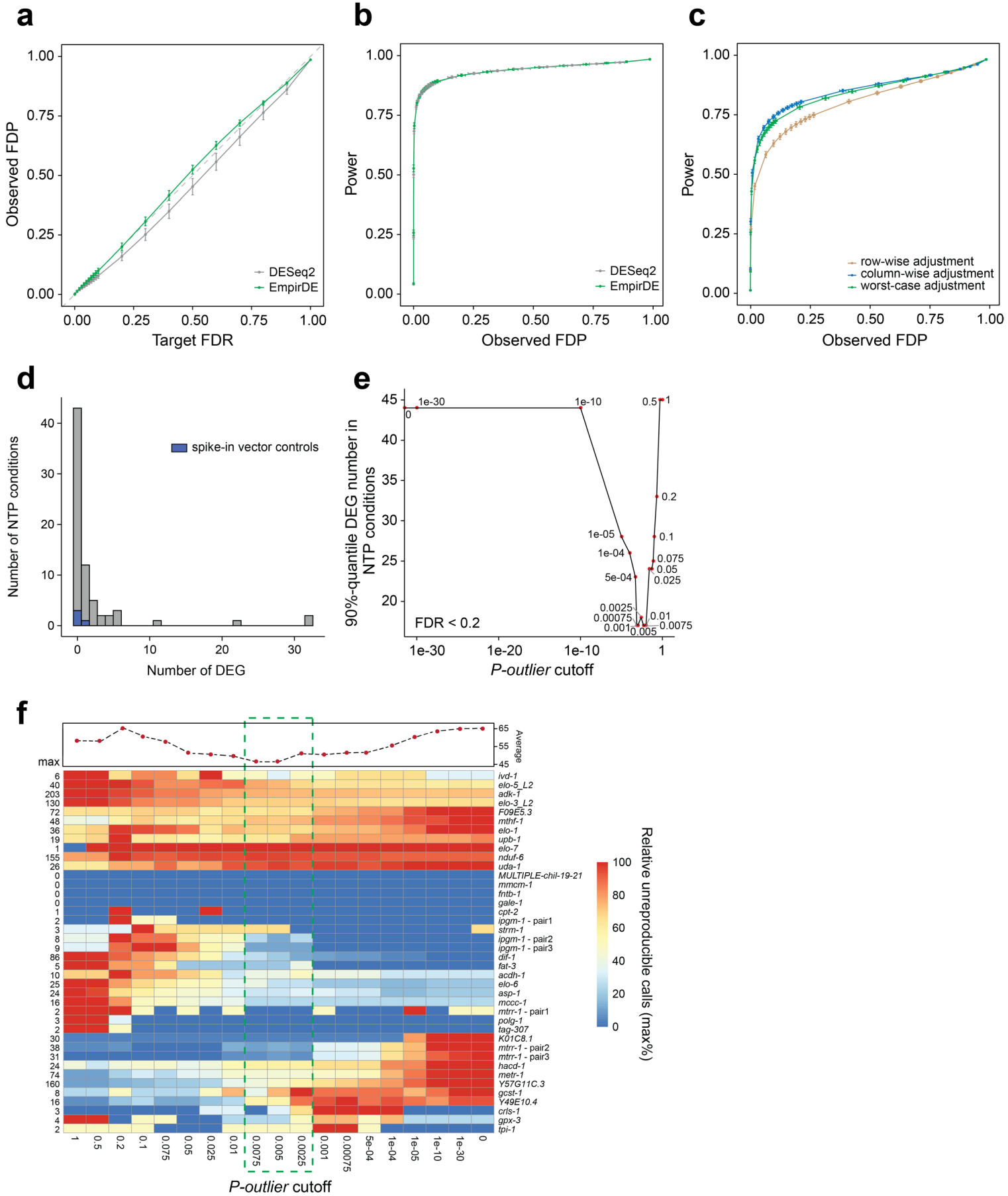
Supplementary Fig. 1: Development of WPS. a, WPS RNA extraction method does not extract RNA from eggs. **b**, Zoom-in heatmap of **Fig. 2a**. **c**, Histogram of gene expression in the reference profile. This reference profile is defined by the first replicate of the

subsampling profiles in **Fig. 2b** (**Supplementary Data 2**). The reference was generated by combining the data of the first replicate from 62 to 65 hours in (**b**). **d**, Read depth distribution of 4,083 WPS samples combining metabolic and NHR WPS datasets. **e,** Number of genes detected at different TPM thresholds. Genes with a TPM greater than the threshold shown in x-axis are deemed as detected. “BGI 25M” refers to an RNA-seq profile of wild-type adult *C. elegans* using a common commercial method (BGI RNA-seq service, an alternative to Illumina TruSeq RNA), with a sequencing depth of ~25 million. “WPS 50M” refers to the reference profile described in (**c**), at a depth of ~50 million and with error bars showing the standard deviations based on three biological replicates. “WPS regular” refers to the standard WPS profiles at a depth of ~6 million, with the curve and error bars showing the average and standard deviations based on 30 random profiles from the metabolic WPS dataset [1]. **f**, Examples of gene expression correlation between replicates with good (left) and poor (right, labeled in red) quality. **g**, Fractions of perturbations with effective targeted gene knockdown versus targeted gene expression. Effective knockdown is defined by a minimum decrease of 1.5 (red), 2 (green) and 3 (blue) folds in the targeted gene expression, based on differential expression analysis. The fraction was calculated using 1,048 valid RNAi conditions from both metabolic and NHR WPS datasets, titrated with a minimum targeted gene expression threshold indicated in x-axis. The Wild-Type (WT) expression was based on the reference profile in (**c**). **h**, Sanger sequencing results of the three fail-QC conditions in **Fig. 2f.** Source data are provided as a Source Data file.

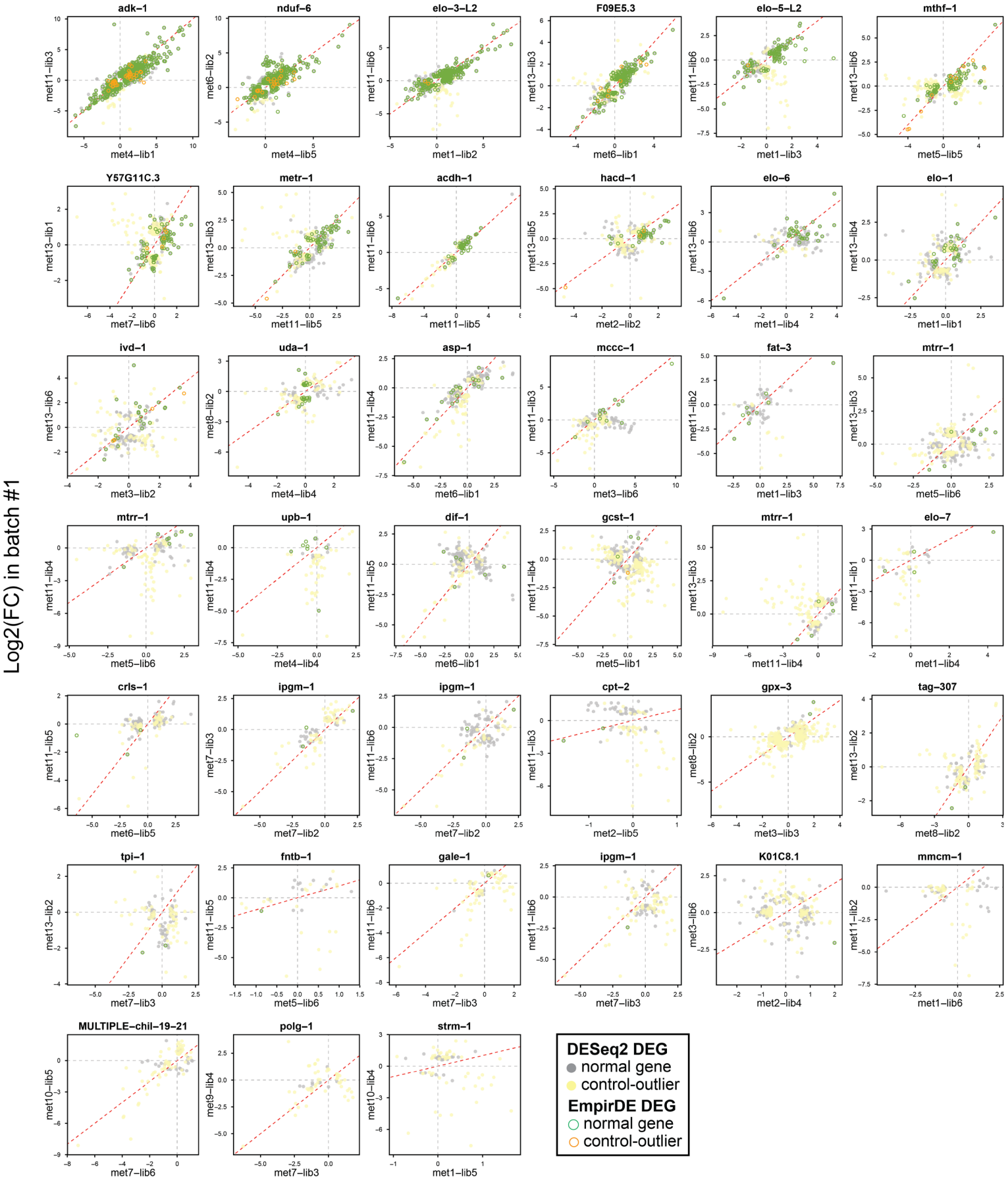


Supplementary Fig. 2: Investigating the source of false discovery in WPS analysis. **a,** Scatter plot showing the expression level of control-outlier genes in different types of samples. The control-outlier genes in the 72 metabolic-gene WPS sequencing libraries are combined and displayed in the plot and each data point refers to a control-outlier gene

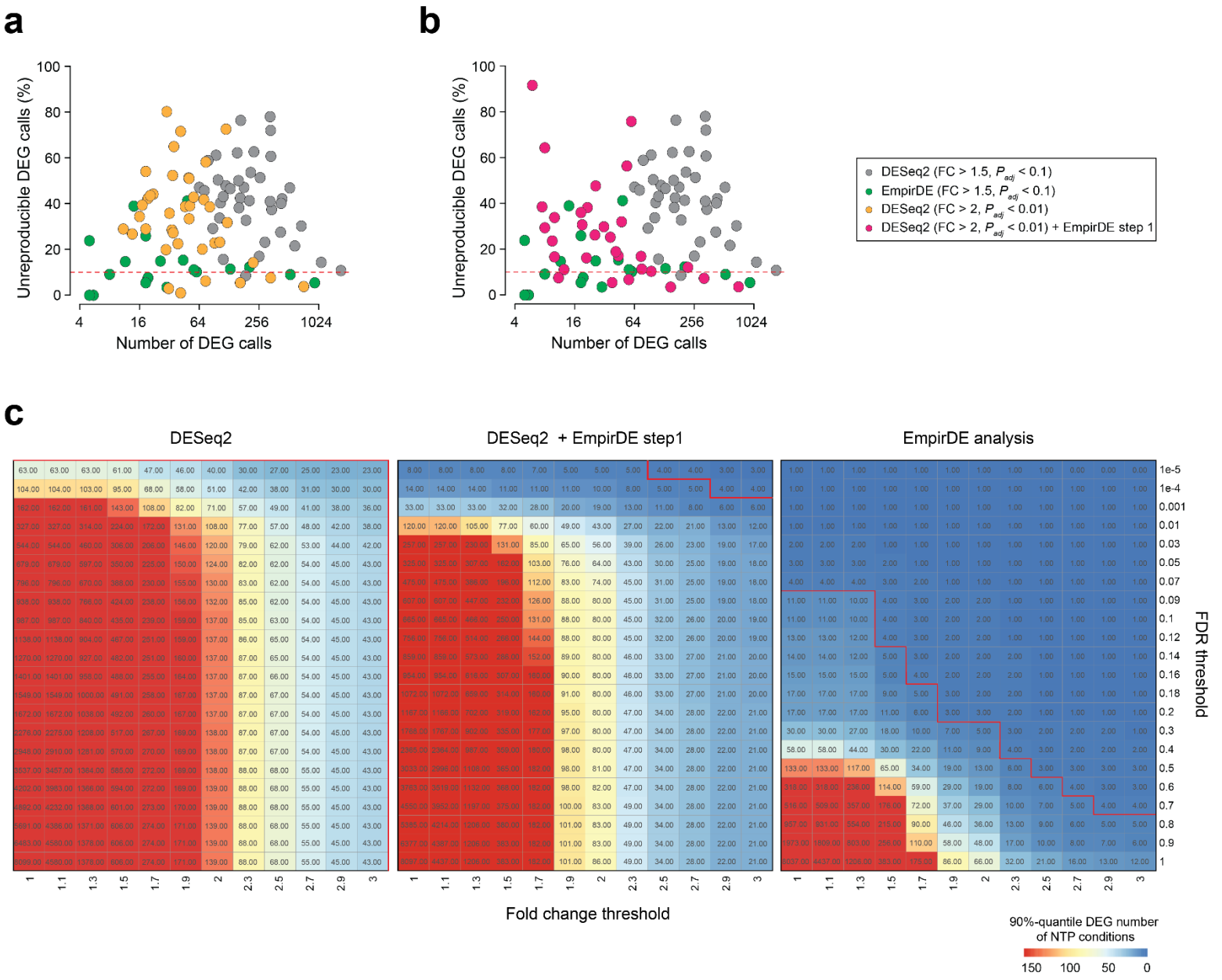
in a sequencing library. The y-axis represents the average TPM of all RNAi samples (excluding NTPs and vector controls) in a sequencing library, which is compared to the average TPM of either NTP (red) or vector control (blue) samples in the same sequencing library on the x-axis. **b**, Gene expression levels of two example genes exhibiting high (top) or moderate (down) fluctuation of the mean. The three panels from left to right present a sequential zoom-in view of the expression pattern at the level of entire metabolic-gene WPS dataset, an RNAi plate, and a sequencing library. The perturbation index is an arbitrary index indicating the number of perturbations displayed in the plot. **c**, Illustration of the quantification and simulation of random fluctuation of the mean. To quantify the fluctuation level of each gene, a distribution of its log2(FC) across the thousand RNAi conditions in the metabolic-gene WPS dataset was fitted by the same procedure used for fitting the empirical null of the Wald statistic. The fitted sigma from real data was compared to that from the simulation data without $\Delta\mu$. Inflation of the sigma level compared with simulation fit was used as a proxy of mean fluctuation to create a heuristic (formula on the right) for generating gene-specific random $\Delta\mu$ (**Supplementary Methods**). **d**, **e**, Comparison of the fitted standard deviation of the Wald statistic between simulation and real data for all genes. The red dashed line indicates the diagonal (y=x). The scatter plot shows results from one simulation of the metabolic-gene WPS dataset. **f**, Similar to (**e**), but showing the average of fitted standard deviations in 10 independent simulations.


a
Observed FDP
Target FDR
DESeq2
EmpirDE
b
Power
Observed FDP
DESeq2
EmpirDE
c
Power
Observed FDP
row-wise adjustment
column-wise adjustment
worst-case adjustment
d
Number of NTP conditions
Number of DEG
spike-in vector controls
e
90%-quantile DEG number in NTP conditions
P-outlier cutoff
FDR < 0.2
f
max
Average
P-outlier cutoff
Relative unreproducible calls (max%)
ivd-1
elo-5_L2
adk-1
elo-3_L2
F09E5.3
mthf-1
elo-1
upb-1
elo-7
nduf-6
uda-1
MULTIPLE-chil-19-21
mmcm-1
fntb-1
gale-1
cpt-2
ipgm-1 - pair1
strm-1
ipgm-1 - pair2
ipgm-1 - pair3
dif-1
fat-3
acdh-1
elo-6
asp-1
mccc-1
mtrr-1 - pair1
polg-1
tag-307
K01C8.1
mtrr-1 - pair2
mtrr-1 - pair3
hacd-1
metr-1
Y57G11C.3
gcst-1
Y49E10.4
crls-1
gpx-3
tpi-1

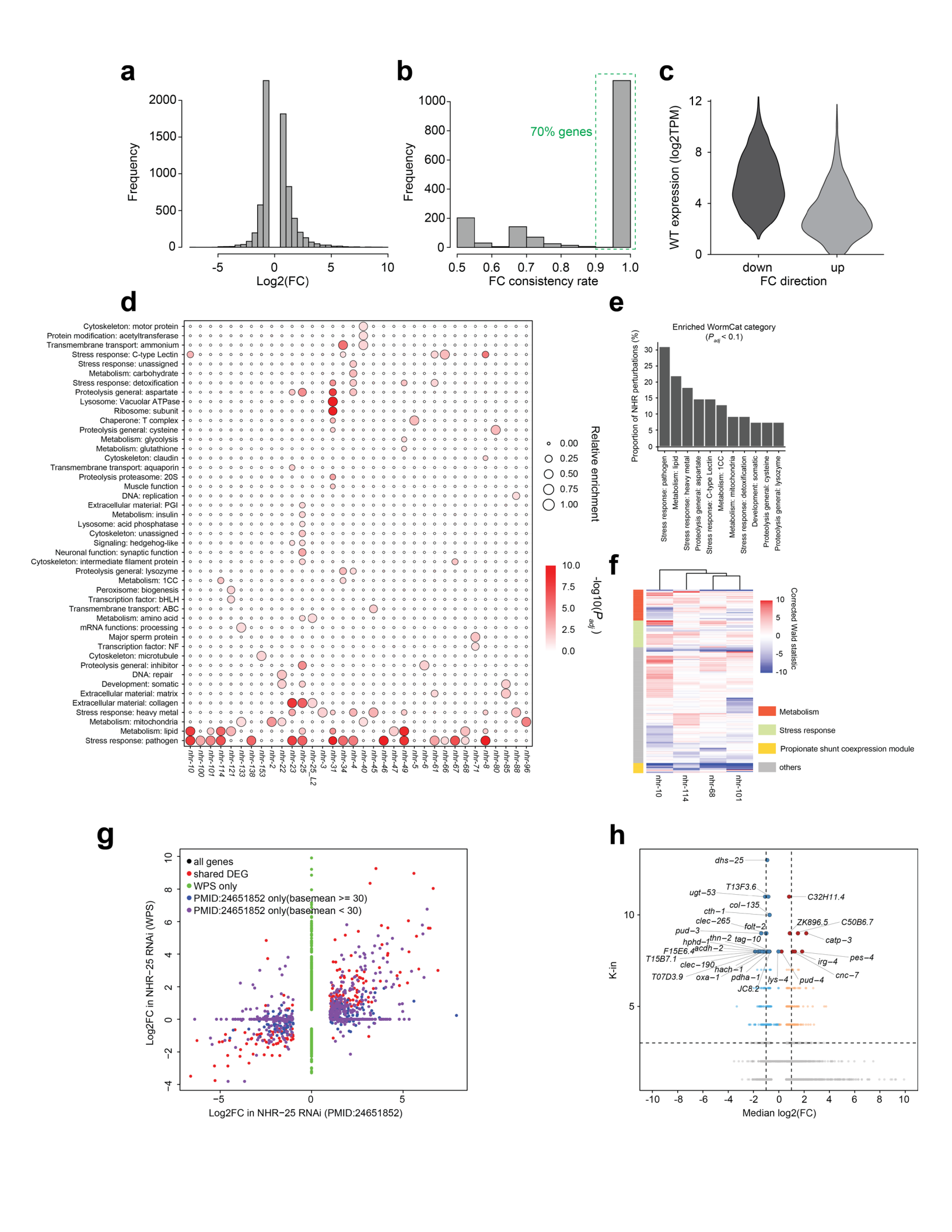
Supplementary Fig. 3: Benchmarking and parameter optimization of EmpirDE analysis framework. a, **b**, Performance benchmarking of the EmpirDE analysis using simulation data without $\Delta\mu$. FDP, false discovery proportion. **c**, Benchmarking the FDR control of different multiple testing adjustment strategies. **d**, Distribution of the number of DEGs in non-targeting perturbations (NTPs) identified by EmpirDE (FC > 1.5, FDR < 0.1). **e, f,** Two evaluations of false discovery to select the threshold for defining control-outlier genes in the first step of EmpirDE analysis. (**e**) shows the false discovery estimation by DEG calls in NTP samples across different *P*-outlier thresholds, and (**f**) shows the estimation by unreproducible calls in the 36 repeated conditions. To increase the sensitivity of the analysis, DEGs in (**e**) were solely defined with FDR threshold of 0.2. Colors in (**f**) show the fraction of all unreproducible DEGs with respect to the maximum level in each condition across a range of *P-outlier* threshold. The maximum levels of unreproducible DEGs are shown on the left of the heatmap. Scatter plot on the top shows the column-wise average (only conditions with maximum level greater than 5 DEGs were used for better robustness). For definition of unreproducible DEGs, please refer to **Supplementary Methods**.


adk−1
nduf−6
elo−3_L2
F09E5.3
elo−5_L2
mthf−1
Y57G11C.3
metr−1
acdh−1
hacd−1
elo−6
elo−1
ivd−1
uda−1
asp−1
mccc−1
fat−3
mtrr−1
mtrr−1
upb−1
dif−1
gcst−1
mtrr−1
elo−7
crls−1
ipgm−1
ipgm−1
cpt−2
gpx−3
tag−307
tpi−1
fntb−1
gale−1
ipgm−1
K01C8.1
mmcm−1
MULTIPLE−chil−19−21
polg−1
strm−1
DESeq2 DEG
normal gene
control−outlier
EmpirDE DEG
normal gene
control−outlier
Log2(FC) in batch #1
Log2(FC) in batch #2

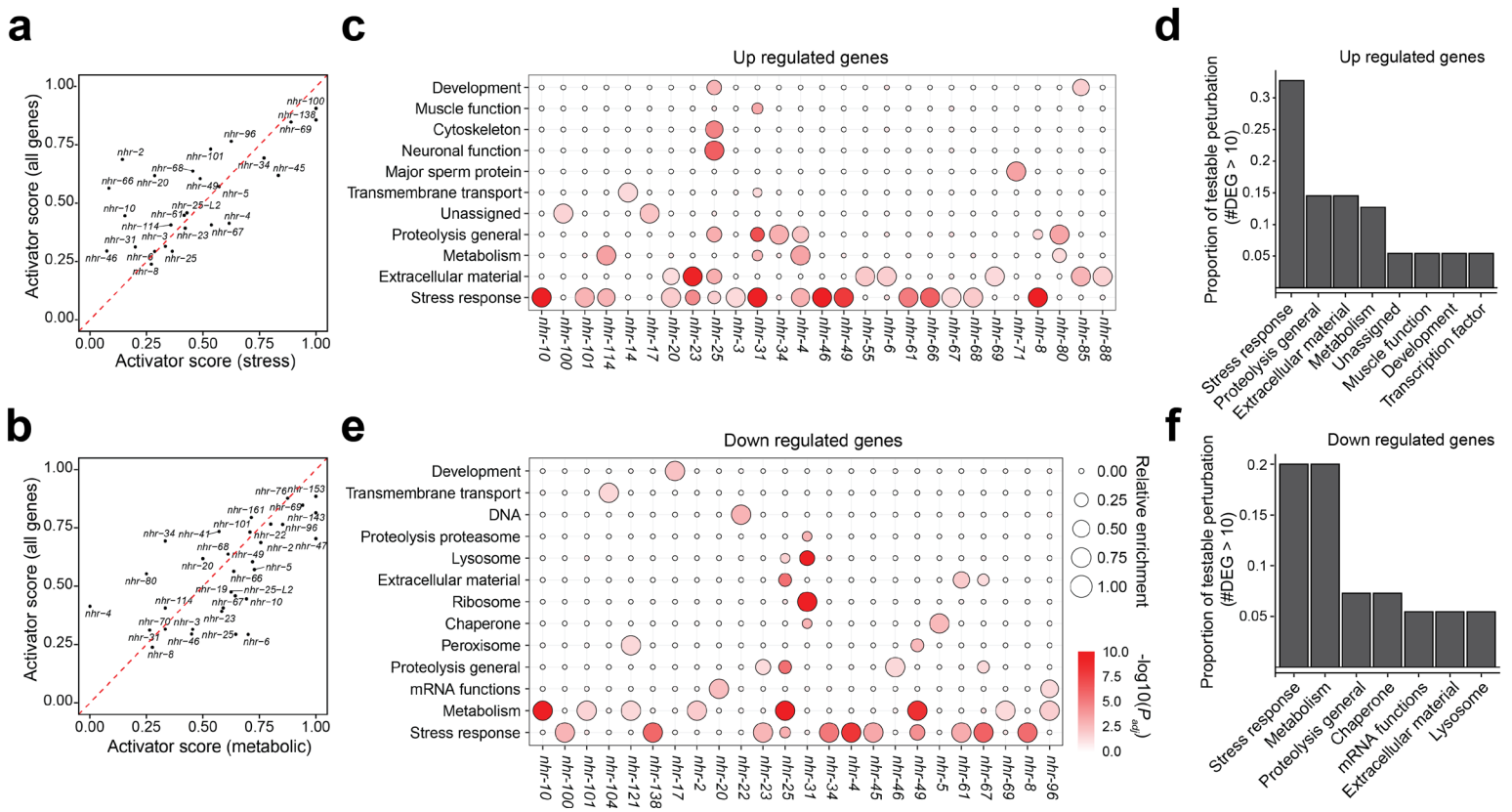
Supplementary Fig. 4: WPS analysis reproducibility. Thirty-six WPS experiments were repeated, and some were done more than twice (e.g., *mtrr-1*). Each experiment included triplicate perturbations. Plots show comparison of the log2(FC) of genes called as DEGs (FC > 1.5 and FDR < 0.1) in either experiment. Red dashed lines indicate the diagonal (y=x).



Supplementary Fig. 5: Benchmark analysis of DESeq2 with additional treatments. **a**, **b,** Fraction of unreproducible DEGs in DESeq2 analysis with additional treatments. Unreproducible DEGs were defined as in **Fig. 4f**. (**a**) compares the results between regular DESeq2 analysis (grey), EmpirDE (green) and DESeq2 with more conservative thresholds (yellow). (**b**) shows a similar comparison but with the first step of EmpirDE applied in DESeq2 analysis. (**c**) Evaluating false discoveries using NTPs. The results from DESeq2 plus the first step of EmpirDE (middle) is compared with the results shown in **Fig. 4d** (left and right).


a
Frequency
Log2(FC)
b
70% genes
Frequency
FC consistency rate
c
WT expression (log2TPM)
down
up
FC direction
d
Cytoskeleton: motor protein
Protein modification: acetyltransferase
Transmembrane transport: ammonium
Stress response: C-type Lectin
Stress response: unassigned
Metabolism: carbohydrate
Stress response: detoxification
Proteolysis general: aspartate
Lysosome: Vacuolar ATPase
Ribosome: subunit
Chaperone: T complex
Proteolysis general: cysteine
Metabolism: glycolysis
Metabolism: glutathione
Cytoskeleton: claudin
Transmembrane transport: aquaporin
Proteolysis proteasome: 20S
Muscle function
DNA: replication
Extracellular material: PGI
Metabolism: insulin
Lysosome: acid phosphatase
Cytoskeleton: unassigned
Signaling: hedgehog-like
Neuronal function: synaptic function
Cytoskeleton: intermediate filament protein
Proteolysis general: lysozyme
Metabolism: 1CC
Peroxisome: biogenesis
Transcription factor: bHLH
Transmembrane transport: ABC
Metabolism: amino acid
mRNA functions: processing
Major sperm protein
Transcription factor: NF
Cytoskeleton: microtubule
Proteolysis general: inhibitor
DNA: repair
Development: somatic
Extracellular material: matrix
Extracellular material: collagen
Stress response: heavy metal
Metabolism: mitochondria
Metabolism: lipid
Stress response: pathogen
nhr-10
nhr-100
nhr-101
nhr-114
nhr-121
nhr-133
nhr-138
nhr-153
nhr-2
nhr-22
nhr-23
nhr-25
nhr-25_L2
nhr-3
nhr-31
nhr-34
nhr-4
nhr-40
nhr-45
nhr-46
nhr-47
nhr-49
nhr-5
nhr-6
nhr-61
nhr-66
nhr-67
nhr-68
nhr-71
nhr-8
nhr-80
nhr-85
nhr-88
nhr-96
Relative enrichment
0.00
0.25
0.50
0.75
1.00
-log10(Padj)
10.0
7.5
5.0
2.5
0.0
e
Enriched WormCat category
(Padj < 0.1)
Proportion of NHR perturbations (%)
Stress response: pathogen
Metabolism: lipid
Stress response: heavy metal
Proteolysis general: aspartate
Stress response: C-type Lectin
Metabolism: 1CC
Metabolism: mitochondria
Stress response: detoxification
Development: somatic
Proteolysis general: cysteine
Proteolysis general: lysozyme
f
Corrected Wald statistic
Metabolism
Stress response
Propionate shunt coexpression module
others
nhr-10
nhr-114
nhr-68
nhr-101
g
all genes
shared DEG
WPS only
PMID:24651852 only(basemean >= 30)
PMID:24651852 only(basemean < 30)
Log2FC in NHR−25 RNAi (WPS)
Log2FC in NHR−25 RNAi (PMID:24651852)
h
dhs−25
T13F3.6
ugt−53
C32H11.4
col−135
cth−1
clec−265
ZK896.5
C50B6.7
folt−2
pud−3
catp−3
hphd−1
thn−2
tag−10
acdh−2
F15E6.4
T15B7.1
irg−4
pes−4
clec−190
hach−1
cnc−7
T07D3.9
oxa−1
pdha−1
lys−4
pud−4
JC8.2
K-in
Median log2(FC)

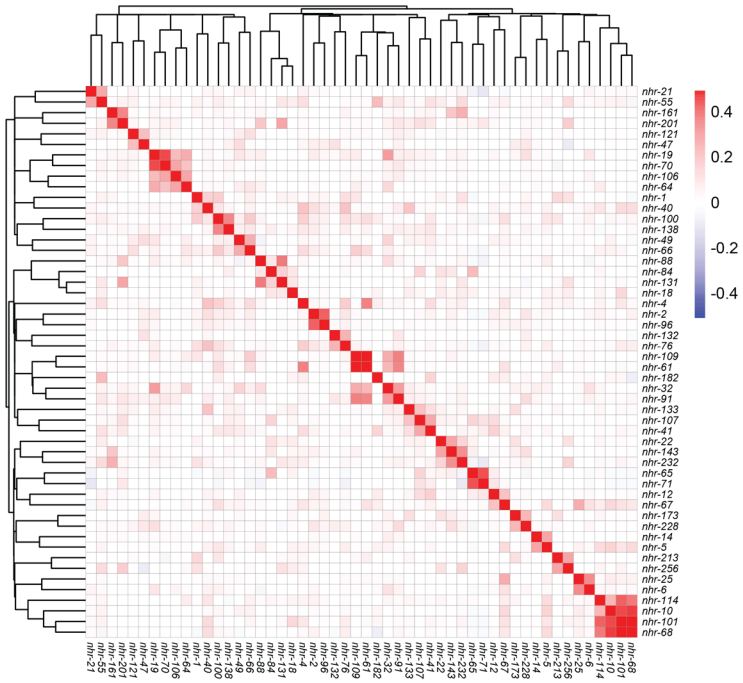
Supplementary Fig. 6: Analysis of NHR WPS data. a, Log2(FC) distribution of all DEGs in the NHR WPS experiments. **b**, Distribution of the fold change consistency (up or down) for all DEGs with k-in ≥ 2, which was defined as the proportion of DEG calls that increase or decrease in the WPS experiments (whichever greater). **c**, Association between fold change direction and basal expression level of the DEGs. **d**, Gene set enrichment analysis of NHR perturbations using WormCat Level 2 categories[2]. Only NHR perturbations with more than 10 DEG calls were analyzed. Conditions without significant (P_{adj} > 0.05) enrichment are not shown in the plot. The relative enrichment is defined as the -log10(P_{adj}) normalized by its maximum in a given perturbation. **e,** Proportion of testable perturbations that showed significant enrichment (P_{adj} < 0.1) in WormCat Levels 2 categories. **f**, Heatmap displaying the corrected Wald statistics for the union of DEGs in *nhr-10*, *nhr-68*, *nhr-101*, and *nhr-114* perturbations. The propionate shunt coexpression module was defined based on a previous study[3]. **g,** Comparison between WPS data and public data for *nhr-25* knockdown. The 'WPS only' group (green dots) indicates genes that are called as DEGs in WPS data but not available in the referred study. Some of the differences may stem from discrepancies in the developmental stage of the worms (L4 stage in [4] and adult stage for our data), or differences in gene detection (RNA-seq vs. microarray). **h,** Volcano plot showing the hub DEGs in the NHR GRN. The median log2(FC) is the median level of the log2(FC) values of all interactions for a corresponding gene in the GRN. The red and blue colors are used to indicate up and down regulated hub DEGs, respectively. Source data are provided as a Source Data file.



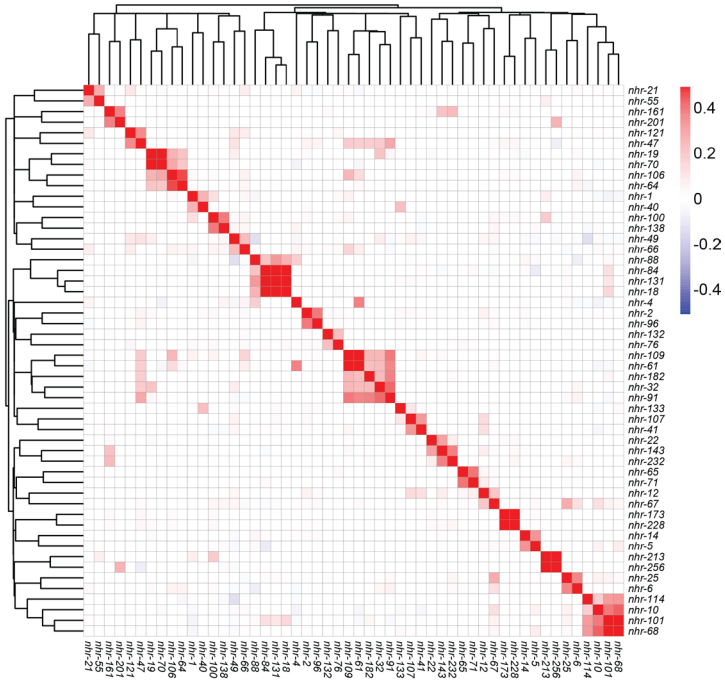
Supplementary Fig. 7: Analysis of NHR GRN. a, b, Correlation between the activator score for all DEGs and that for stress response (**a**) and metabolic (**b**) genes. The stress response and metabolic genes were defined based on WormCat Level 1 categories[2]. Only perturbations with ≥ 5 stress (**a**) or metabolic (**b**) DEGs are shown in the plot. **c, d**, **e**, **f**, Gene set enrichment analysis of up-regulated (**c**,**d**) and down-regulated (**e**,**f**) genes. WormCat Level 1 categories were used as the gene sets. Source data are provided as a Source Data file.

Supplementary heatmaps of perturbation-perturbation similarity

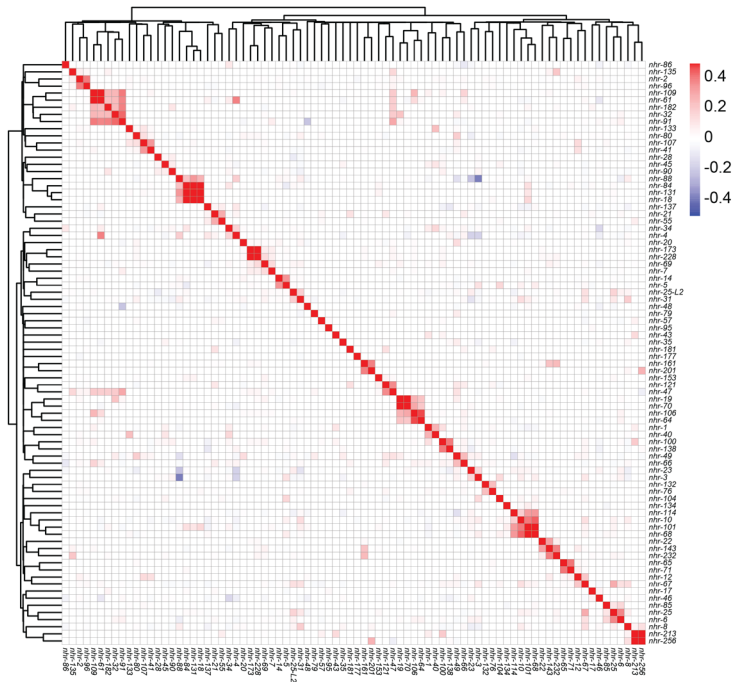
52 selected NHR perturbations
(by DESeq2 analysis)



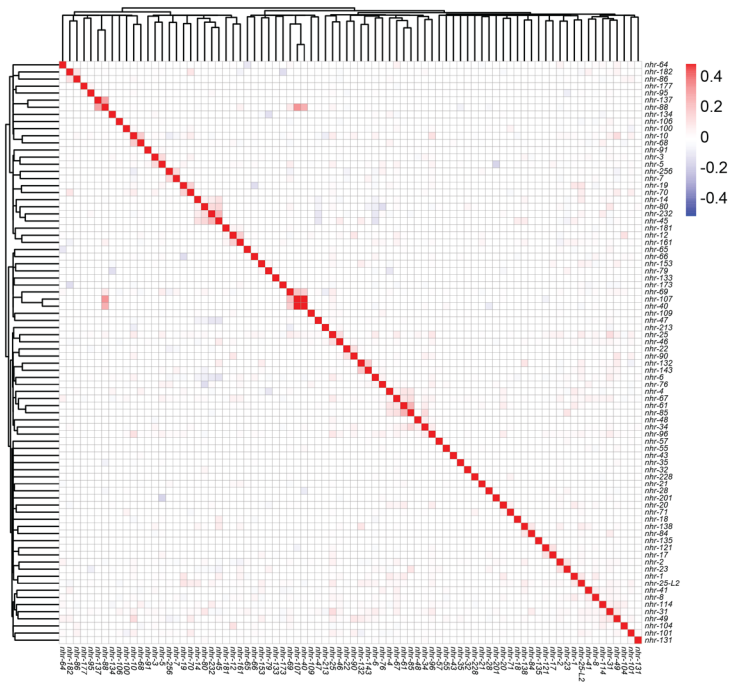
52 selected NHR perturbations
(by EmpirDE analysis, also in **Fig. 6a**)



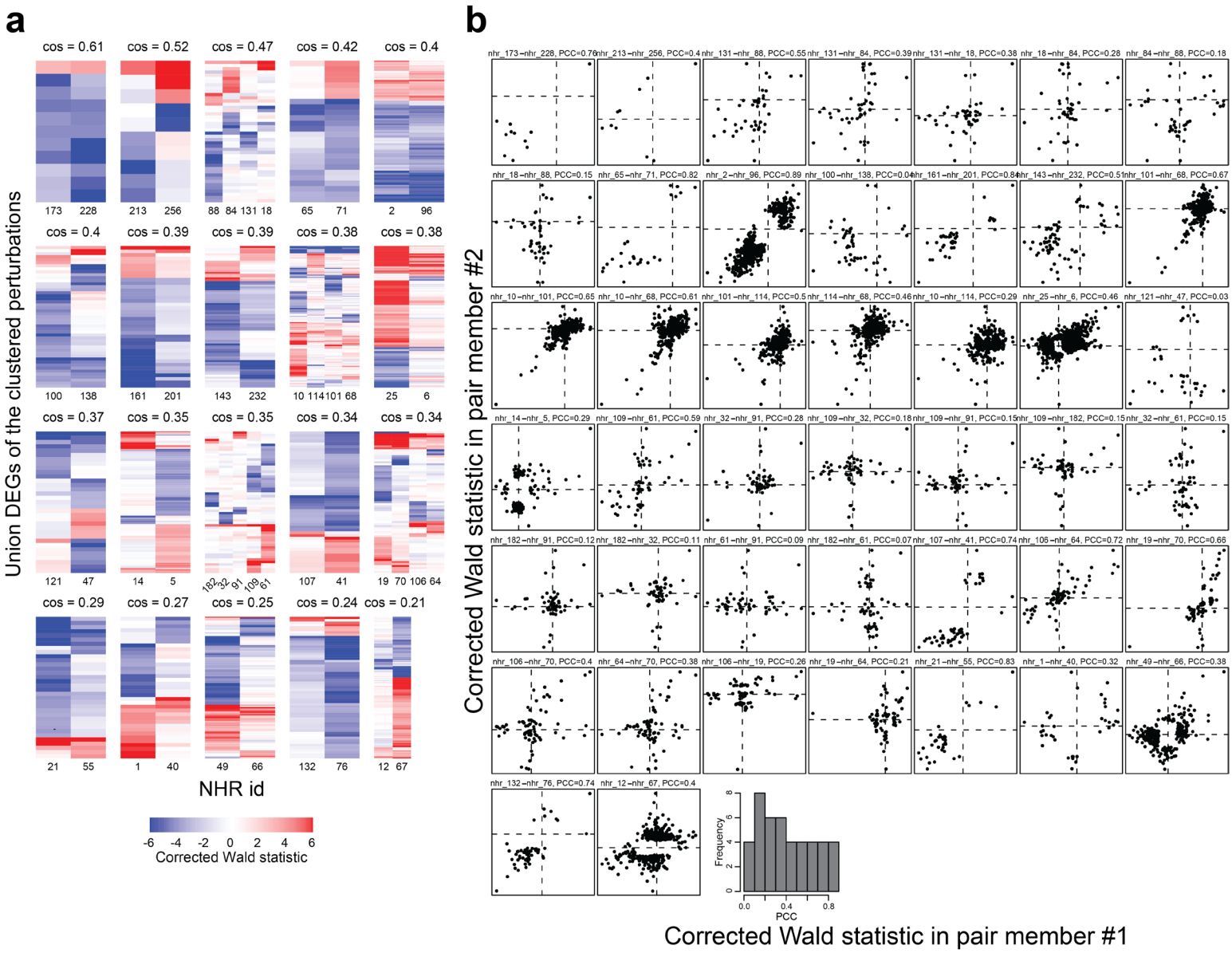
All 82 NHR perturbations (81 NHR genes)
(by EmpirDE analysis)



All 82 NHR perturbations (81 NHR genes)
(randomized NHR GRN)

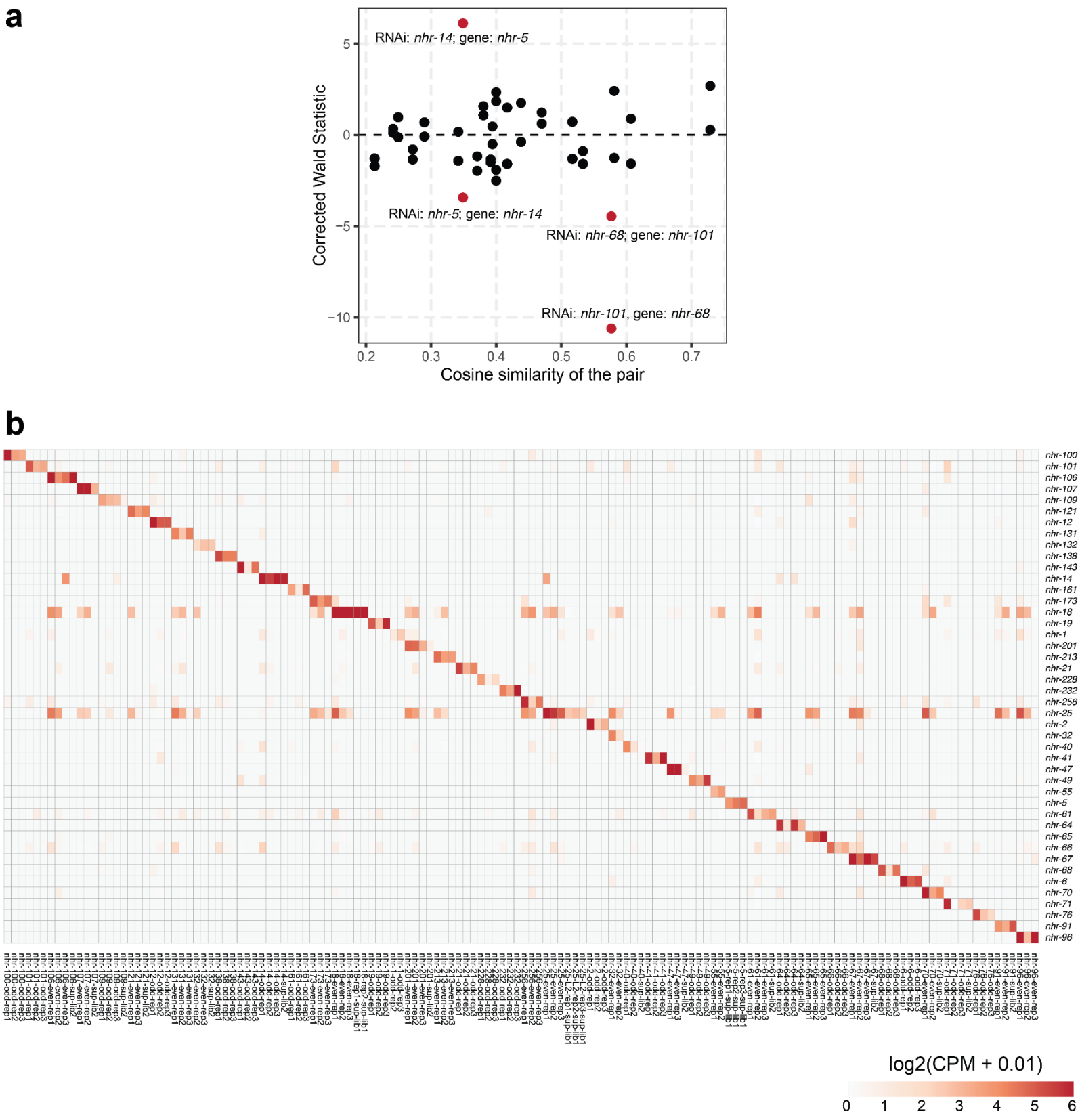


Supplementary Fig. 8: Supplementary heatmaps of perturbation-perturbation similarity.



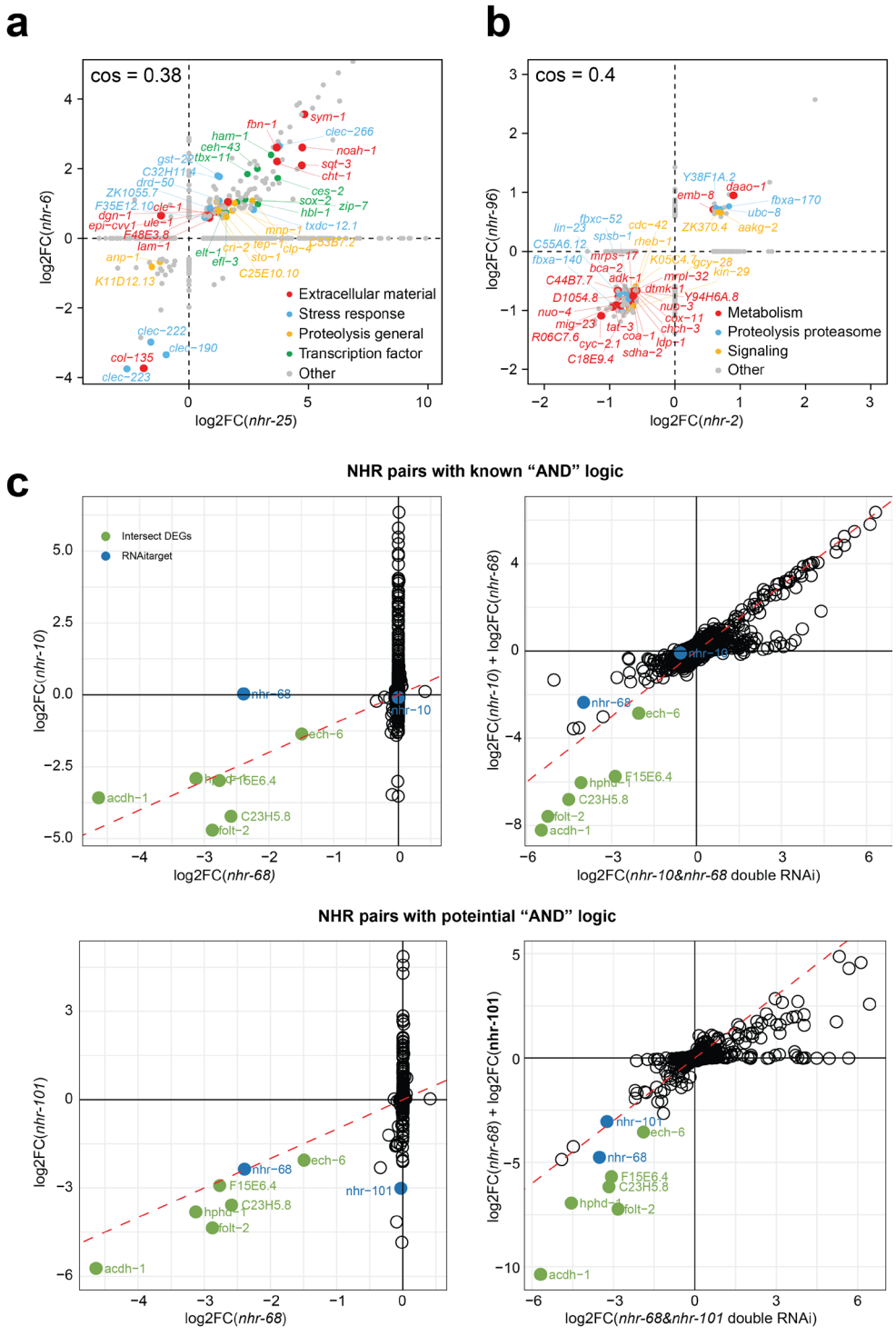
Supplementary Fig. 9: Visualization of the gene expression changes of selected NHR perturbation clusters. a, Heatmaps showing the corrected Wald statistic. The NHR perturbations were grouped by clusters. These clusters were identified by cutting the hierarchical tree in **Fig. 6a** using *cutree* function in R with h = 0.9. The union DEGs of conditions in each cluster are shown in the y-axis. The numbers in x-axis indicate the number in the corresponding *nhr* gene name. cos, cosine similarity. When there are more than two conditions in a cluster, the average pairwise cosine similarity was labeled. **b**, Scatter plots showing the correlation of expression changes in NHR pairs. The NHR pairs were defined as the intra-cluster pairs in (**a**) and the corrected Wald statistics for the union

DEGs of the pair were shown in the plot. A histogram for the Pearson Correlation Coefficient (PCC) of each pair is shown at the bottom.



Supplementary Fig. 10: Gene expression changes and anti-sense RNA signals of the counterpart NHR. a, Expression changes of the counterpart NHR gene upon the

perturbation of the other member of a pair. The NHR pairs were defined by the tree cutting approach described in **Fig. 6c**, with a minimal cosine similarity of 0.21. The gene expression changes are visualized by the corrected Wald statistic and significant changes (FDR < 0.1, FC > 1.5) are labeled in red. In general, we found that the expression levels of the counterpart NHR were not affected. Interestingly, we noticed two pairs of NHRs presenting clear inter-regulation: perturbation of *nhr-14* up regulates its counterpart *nhr-5*, while the perturbation of *nhr-5* down regulates *nhr-14*. In addition, perturbations of *nhr-68* and *nhr-101* downregulates the expression of each other. While the latter case may indicate an off-target effect, we think it is unlikely because the mRNA sequences of these two genes do not contain long identical regions (overall percentage identity = 71%). In addition, we confirmed that there are no signs of cross-contamination among the NHR pairs based on dsRNA signals in (**b**). Therefore, we conclude that the pairwise modularity is not driven by technical artifacts such as off targeting or cross contaminations. **b**, dsRNA quantification for all NHR genes presenting pairwise modularity. The genes and perturbations are shown in rows and columns, respectively, and are clustered in accordance with each other such that the diagonal indicates the dsRNA signals for the perturbed gene.



Supplementary Fig. 11: Visualization of the gene expression changes of selected NHR perturbation pairs. a, **b**, Comparison of filtered log2(FC) profile for two example pairs of NHR perturbations. The union set of DEGs in the two conditions are shown in the plot. cos, cosine similarity. **c,** The scatter plots show the gene expression changes in the

perturbations of two pairs of NHRs. Two plots on the left visualize the log2(FC) values in the single-gene perturbations, with shared DEGs labeled in green. The plots on the right compare the observed log2FC with expected levels based on additive model (the sum of log2FC in the two single perturbations). Red dashed line indicates the diagonal. The observed FCs of shared DEGs are substantially lower than the additive expectation (below the diagonal), consistent with the AND logic in which perturbing the second gene does not have additional effects. Source data are provided as a Source Data file.

Supplementary Notes

Supplementary Note 1: Overview of RNAi bacterial library preparation, RNA extraction and multiplexed sequencing library construction for Worm Perturb-Seq (WPS)

To start an WPS experiment, an RNAi bacterial library was prepared for genes of interest, each from a single colony of bacteria harboring the corresponding RNAi construct. In each WPS experiment, RNAi bacteria were grown to log phase before being seeded in 6-well solid-media plates. Bacterial expression of double stranded RNA for RNAi was induced by Isopropyl β-D-1-thiogalactopyranoside (IPTG) and ~200 *C. elegans* at the first larval stage (L1) were added to each well. When grown to the desired stage, animals were harvested in a 96-well plate for RNA extraction.

We developed a protocol for the high-throughput RNA extraction in 96-well plates (**Supplementary Protocol**). The most frequently used methods for RNA extraction use Trizol and multiple liquid nitrogen freeze/thaw cycles to break the animal's cuticle in individual tubes, which does not enable easy high-throughput experiments. Therefore, we explored whether we could use a combination of SDS and DTT, which has previously been used to break the animal's cuticle[5]. We used this method in combination with an optimized chemical cocktail containing proteinase K, commonly used worm lysis buffer[6], and a commercially available tissue lysing reagent to lyse the animal in 96-well plates. Importantly, this approach does not lyse eggs, making it suitable to use WPS for gravid adults (**Supplementary Fig. 1a**). After lysis, RNA was extracted using 96-well silica columns[7] (**Supplementary Protocol**). Finally, RNA concentrations were normalized to facilitate even pooling of samples in the next step.

To generate multiplexed RNA-seq libraries, we optimized the 3' end barcoding method CEL-Seq2[8], which was originally developed for single-cell RNA-seq[9]. We optimized the protocol for bulk RNA-seq application by reducing the use of costly reagents, such as reverse transcriptase, and found that a general 10x reduction produced libraries with similar quality and yield. We also optimized other experimental details such as incubation times, substrate concentrations, Quality Control (QC) checkpoints, and an earlier pooling of samples (**Fig. 1b** and **Supplementary Protocol**).

Supplementary Note 2: control-outlier genes and control-independent DE analysis

In the first step of EmpirDE, we performed Differentially Expression (DE) analysis at the level of individual sequencing libraries that consisted of ~16 conditions in triplicates (**Fig. 3d**, top). We first identified control-outlier genes as those consistently up- or down-regulated in at least three-quarters of conditions in a sequencing library, using a *P* value threshold (see **Supplementary Methods**). We found that most sequencing libraries contained fewer than 200 control-outlier genes (**Fig. 3e**). Importantly, albeit being consistently changed when compared with vector controls, the expression levels of these control-outlier genes in RNAi conditions were not systematically shifted when compared with the NTP condition(s) in the same sequencing library (**Supplementary Fig. 2a**). This validated that it was the gene expression in the vector control samples that was confounded. For these genes, we developed a control-independent DE analysis (**Fig. 3d,** top, algorithmic details are in **Supplementary Methods**). Briefly, for each control-outlier gene, we empirically identified a control-independent null population based on the distribution of its expression levels in a sequencing library. We applied an algorithm used by the GTEx consortium[10] to fit the distribution and to distinguish the main population that

includes most conditions from outliers. Assuming the gene expression is not affected by most perturbations, this main population of the gene expression levels can be used as a null (**Fig. 3d**). We then performed DESeq2 by comparing the level for the gene in each RNAi condition to the newly defined null level. If a gene frequently appears as a control-outlier in different sequencing libraries (*i.e.,* > 25% of the libraries), we performed control-independent DE analysis for this gene in all libraries. For most regular, non-control-outlier genes, we used regular DESeq2 analysis, *i.e.,* compared each RNAi condition with the vector control from the same sequencing library to calculate the DE statistics (**Fig. 3d**).

Supplementary protocol: Worm Perturb-Seq (WPS)

Section I: Animal culture and RNA interference

RNA interference library

First, construct WPS RNAi libraries from parent libraries, such as the metabolic[11], TF[12] or ORFome[13] libraries. We recommend constructing a working library for each WPS study using single colonies from the parent library. Specifically, streak the corresponding bacteria onto LB agar plates supplemented with 50 μg/mL ampicillin to allow the formation of single colonies. Pick one colony per RNAi condition, grow the bacteria in 1 mL of LB medium containing 50 μg/mL ampicillin in a 96-deep well plate, and make glycerol stocks in 96-well plates as the WPS RNAi working library.

To reduce the risk of being contaminated, we recommend avoiding placing vector control bacteria directly in the 96-well plates of WPS working library. Instead, make a separate glycerol stock in cryopreservation tube for vector control bacteria. In each WPS experiment, re-introduce the vector control at the step of LB liquid culture alongside RNAi bacteria, either in the same or a different 96-well plate. The details of this process will be discussed in the following section.

RNA interference

RNAi of WPS follows a previously described protocol with slight modifications[14]. Specifically, inoculate bacteria from the WPS working library into a 96-deep well plate with 1 mL of LB medium containing 50 μg/mL ampicillin and shake overnight at 37°C. Dilute 100 uL of each overnight culture by 50-fold using fresh LB medium containing 50 μg/mL ampicillin in a well of a 24-deep well plate and shake for 4 hours at 37°C. After incubation, collect the bacteria by centrifuging the plate at 3,000 g for 20 minutes. Next, resuspend the resulting pellets in 200 μL of M9 solution and transfer to 6-well NGM plates containing 50 μg/mL ampicillin and 2 mM Isopropyl β-d-1-thiogalactopyranoside (IPTG, Fisher Scientific) to induce the expression of RNAi dsRNA. Allow the plates to dry in a hood and then sit on a bench table overnight at room temperature. The RNAi plates are now ready to use for plating worms (see below).

IMPORTANT: Best practice in WPS experimental design

During the course of our experiments we discovered several issues that would affect future WPS experimental design. First, it would be advised to minimize batch effects by keeping control and treatment samples in the same batch whenever possible. This applies to all stages of the experiment, including but not limited to culturing bacteria on the same plate, using the same incubator, and pooling samples in the same sequencing library. However, strict batch control may need to be adjusted for practical reasons. One critical consideration is bacterial culturing, for which we provide specific recommendations below.

As mentioned earlier, we observed that the NHR WPS library had a significantly lower incidence of control-outlier genes compared to metabolic WPS (~10-fold fewer, data not shown). We suspect a relevance to the preparation of bacterial diet: for NHR WPS,

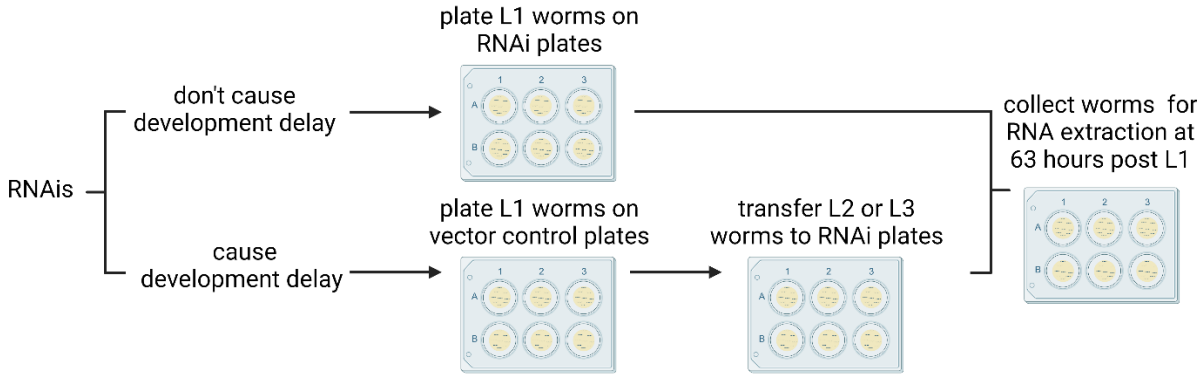
control bacteria were cultured in the same 96-well plate with the RNAi bacteria. However, for metabolic WPS, control bacteria were cultured in a second 96-well plate because of the inclusion of more RNAi conditions in each experiment. This separation was necessary because ~10-15% of the RNAi clones are expected to cause a growth phenotype in large screens (like in metabolic gene WPS). These conditions can be reassessed by a delayed seeding scheme (see below). However, to always proceed with 96 WPS samples with normal development in each batch of experiment, we opted to include additional RNA conditions in the experiment. For future users, we recommend two options for optimizing your WPS experimental design:

The first option is to start with more than 96 RNAi clones (e.g., 120), by culturing a second plate of RNAi bacteria, as what was done in our metabolic WPS screen. However, a substantial batch effect was discovered with this setup, and users are encouraged to follow our experimental design and computational procedure to eliminate this effect. First, the vector control samples should be cultured in the second RNAi plate, which contains fewer RNAi clones (the ~20 additional clones). This setup ensures the primary population of RNAi conditions is derived from the first plate, forming the null population essential for control independent differential expression analysis to eliminate control outlier genes. Subsequently, users should follow the EmpirDE pipeline for data analysis. If the control bacteria were cultured in the same plate as the main population of RNAi clones, the number of RNAi clones in the second plate would be insufficient to form a null population for control independent differential expression analysis. In such a case, RNAi conditions from the second plate could not be effectively analyzed due to the lack of either same-plate vector control or control-independent null population.

Alternatively, researchers can culture only one 96-well WPS RNAi plate with vector controls in the same plate. In such a case, the batch effect from two plates could be minimized, although it may result in not all WPS samples being sequenced due to the growth phenotype in some samples. As a workaround, a pre-screen can be done to identify and eliminate these phenotypical conditions combined with the delayed seeding scheme, however, at the cost of extra workload.

C. elegans culture

Our WPS protocol uses the N2 *C. elegans* strain. To stage the animals properly, follow the animal culturing scheme as below (**Supplementary Fig. 12**). For most WPS experiments, plate approximately 200 synchronized L1 animals into each well of the seeded 6-well NGM plate (see **RNAi interference** above) and incubate at 20°C for approximately 63 hours before collection. In the cases where RNAi feeding leads to a developmental delay, feed the synchronized L1 animals with vector control RNAi for 17 hours (i.e., to L2 stage) or 25 hours (i.e., to L3 stage); then transfer the animals to corresponding RNAi plates (**Supplementary Fig. 12**). Based on our experience with the metabolic gene RNAi, animals usually develop normally with such delayed RNAi exposure. To perform WPS, only collect animals on which the RNAi treatment does not induce a notable developmental delay.



Supplementary Fig. 12: Staging of WPS animals. Created in BioRender. lee, y. (2025) https://BioRender.com/ylp95vj.

Section II: 96-well plate RNA extraction for *C. elegans*

Although the WPS protocol described here extracts RNA from adult stage animals, this RNA extraction protocol can be applied to *C. elegans* from L2 to gravid adult stages. We have not tested this RNA extraction protocol using L1 staged animals. The number of *C. elegans* per sample needed for different stages are listed below.

Stages	Number of *C. elegans*/sample
L2	>2,500
L3	>1,000
L4 to young adult	>500
Gravid adult	>200

Reagents

1. M9 buffer with 0.01% Triton X-100
2. SDS-DTT solution (200 mM DTT, 0.25% SDS, 20 mM HEPES, pH 8.0, 3% sucrose, stored at −20°C) [5]
3. RNase-free water
4. 100% methanol
5. 100% ethanol
6. 80% ethanol
7. worm lysis buffer [WormBook-Chapter: Reverse genetics. https://www.ncbi.nlm.nih.gov/books/NBK19711/]:
   To make 100 mL worm lysis buffer, mix the following reagents and autoclave:

Components and final concentration	**Stock concentration**	**Qty/100 mL**
50 mM KCL	1 M	5 mL
10 mM Tris pH 8.3	1 M	1 mL
2.5 mM MgCl2	1 M	0.25 mL
0.01% Gelatin		0.01 g
H_2O		87.85 mL

The following are added once the autoclaved solution has cooled:

Components and final concentration	Stock concentration	Qty/100 mL
0.45% NP-40 (IGEPAL)	100% NP-40 (IGEPAL)	0.45 mL
0.45% Tween-20	100% Tween-20	0.45 mL

Store the worm lysis buffer at −20°C and add proteinase K to 1 mg/mL just before use.

8. Monarch® DNA/RNA Protection Reagent [NEB: T2011L]
9. Monarch® RNA Lysis Buffer [NEB: T2012L]
10. Washing buffer 1[7]:
    To prepare 100 mL of Washing Buffer 1, dissolve 11.8 g of Guanidine Thiocyanate (Cat. No. G9277, Sigma) into Rnase-free water to achieve a final volume of 99 mL. Additionally, add 1 mL of 1 M Tris-HCl pH 7.4 (Cat. No. 93313, Sigma) to the mixture. Further pH adjustment is not required. The final concentration of Guanidine Thiocyanate is 1 M now.
11. DNA digestion solution:
    This solution needs to be freshly prepared before each experiment.
    For 8 mL:

Rnase-free water: 7.1 mL
10 X DNA Digestion Buffer [Fisher: E1010-1-16]: 800 µL
Ambion™ Dnase I (Rnase-free) [Thermo Scientific™: AM2222]: 100 µL

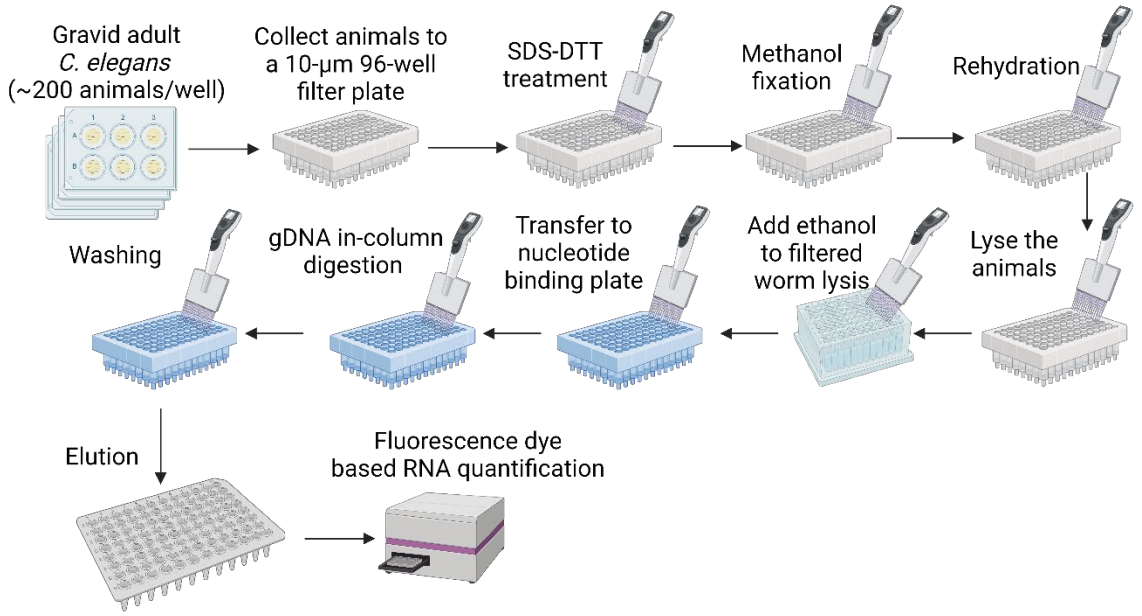
12. 96-Well filter plate (10 µm pore), 960 µL/well [Enzymax: EZ96FTP].
13. 96-Well deep well plate, 2 mL/well [Enzymax: EZ96DWP].
14. 96-Well DNA binding plate, silica membrane [Enzymax: EZ96DBP].
15. AccuBlue® Broad Range RNA Quantitation Kit [Biotium:31073].
16. Greiner Bio-One™ CellStar™ 384-Well, Cell Culture-Treated, Flat-Bottom Microplate [Fisher: 781091].

Equipment

1. Beckman Coulter Avanti® J-26XP High-Performance Centrifuge with a JS-5.3 Swinging-Bucket Centrifuge Rotor.
2. 1 mL, 300 µL and 10 µL multichannel pipettes.
3. Spark® Multimode Microplate Reader

Method

(a graphic breakdown is shown in **Supplementary Fig. 13**)



Supplementary Fig. 13: Overview of 96-well plate RNA extraction protocol for *C. elegans*. Created in BioRender. lee, y. (2025) https://BioRender.com/v67s884.

1. At the time of sample collection (e.g., 63 hours post L1 seeding), wash the animals off the plate with 800 µL of M9 buffer containing 0.01% Triton X-100 and transfer them to a 96-well filter plate. Centrifuge the plate at 2000 g for 2 min and discard the flow-through.
2. Add 100 µL of pre-thawed SDS-DTT solution to each well and incubate at room temperature for 5 min.
3. Add 700 µL of methanol to each well and incubate for 3 min. Centrifuge at 3000 g for 2 min and discard the flow-through. This step is to fix the *C. elegans* tissue and prevent RNA transcriptional changes during RNA extraction.
4. Repeat step 3 two more times.
5. Add 700 µL of H_2O containing 0.01% Triton X-100 to each well and incubate for 3 min. Centrifuge at 3000 g for 1 min and discard the flow-through. Repeat this step two more times.
6. Add 100 µL of pre-thawed worm lysis buffer to each well and incubate at room temperature for 0.5 min.
7. Add 100 µL of Monarch® DNA/RNA Protection Reagent to each well. After adding the reagent, immediately pipette up and down ~20 times. At this point you will see that most tissues are lysed but eggs are still intact when viewed under the microscope. Incubate at room temperature for 5 min. This step is crucial for lysing all *C. elegans* tissue.
8. Add 200 µL of Monarch® RNA Lysis Buffer to each well and incubate at room temperature for 5 min.
9. Put the 96-well filter plate onto a new 96-well deep well plate and centrifuge at 5000 g for 5 min.
10. Add 380 µL of ethanol to the collecting well and transfer liquid to a 96-well DNA

binding plate.
11. Centrifuge at 5000 g for 5 min and discard the flow-through.
12. Add 400 µL of Washing buffer 1 to each well. Centrifuge at 5000 g for 5 min and discard the flow-through.
13. Add 80 µL of DNA digestion solution to each well. Seal the top plate and incubate at 37°C for 15 min.
14. Add 400 µL of Washing buffer 1 to each well. Centrifuge at 5000 g for 5 min and discard the flow-through.
15. Add 400 µL of 80% ethanol to each well. Centrifuge at 5000 g for 5 min and discard the flow-through. Repeat this step one more time.
16. Centrifuge the empty plate at 5000 g for 15 min to completely dry the columns.
17. Add 30 µL of Rnase-free water to each well. Place a 96-well PCR plate on top of the collecting deep well plate and place the DNA binding plate on top of the PCR plate. Centrifuge at 5000 g for 5 min to wash off the RNA. Repeat this step one more time.
18. The elution step usually yields ~30 µL total RNA solution in the PCR plate (the remaining ~30 µL may be retained in the column). Next, measure RNA concentration using the AccuBlue® Broad Range RNA Quantitation Kit in a 384-well plate. Typically, ~30 µL x 200 ng/µL of total RNA can be obtained from each sample.

Section III: WPS sequencing library construction

Reagents

1. Nuclease-Free Water
2. TE buffer (Thermo Fisher Scientific: 12090015)
3. Ethanol
4. Qubit reagents: dsDNA HS Assay (Thermo Fisher Scientific: Q32851)
5. dNTP mix (10 mM each) (Thermo Fisher Scientific: R0192)
6. SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific: 18080044, comes with 0.1 M DTT and First Strand Buffer)
7. RNaseOUT (Thermo Fisher Scientific: 10777019)
8. Second Strand Buffer (Thermo Fisher Scientific: 10812014)
9. DNA Polymerase I (Thermo Fisher Scientific: 18010025)
10. *E. coli* DNA ligase (Thermo Fisher Scientific: 18052019)
11. Ribonuclease H (*E. coli*) (Thermo Fisher Scientific: 18021071)
12. HiScribe™ T7 High Yield RNA Synthesis Kit (NEB: E2040S)
13. Exonuclease I (Thermo Fisher Scientific: EN0581)
14. RNA Fragmentation Reagents (Thermo Fisher Scientific: AM8740)
15. AMPure XP beads (Beckman Coulter: A63880)
16. RNAClean XP beads (Beckman coulter A63987)
17. KAPA HiFi HotStart ReadyMix (Roche: 07958927001)

Equipment

1. Thermocycler
2. Magnetic stand (for 1.5 mL tubes)
3. Qubit® Fluorometer (Thermo Fisher Scientific)

4. Fragment Analyzer (Agilent)

Primers

All our primers maintain the same structure as CEL-Seq2[9], with some differences in the sequences. By using these primers, we can generate sequencing libraries that can be sequenced on both Illumina and BGI platforms.

Barcoding primers

Using primer '1S' (**Supplementary Data 1**) as an example, the barcoding primer has a T7 promoter sequence (highlighted in red) at the beginning, followed by an Illumina adapter sequence (highlighted in yellow; we replaced the original sequence of CEL-Seq2 with the TruSeq adapter sequence), a 7-nt unique molecular identifier (UMI) sequence (NNVNVNV), the same 6-nt unique barcode as CEL-Seq2 (highlighted in green), and a polyT tail ending with 'V'.

All our barcoding primers were purchased from Sangon Biotech using polyacrylamide gel electrophoresis (PAGE) purification. The stock solution for these primers is 10 μM in TE buffer, and the working solution is 2.5 μM in TE buffer. We primarily use the first 60 barcoding primers in our experiments as our sequencing library contains less than 60 samples.

Other primers

The RandomhexRT primer (**Supplementary Table 1**) serves as the primer for the RT2 step (see below) in our protocol. For this step, we have modified the adaptor sequence (highlighted in yellow) to match the 3' end of the Illumina Index PCR Primers.

The P5 primer starts with Illumina P5 sequences (colored in red) and is followed by adaptor sequences that overlap with the adaptor sequences in the barcoding primer. In turn, the index primer begins with the Illumina P7 sequence and is followed by a 6-nt index sequence (highlighted in green) and adaptor sequences (highlighted in yellow) that overlap with the adaptor sequences in the RandomhexRT primer.

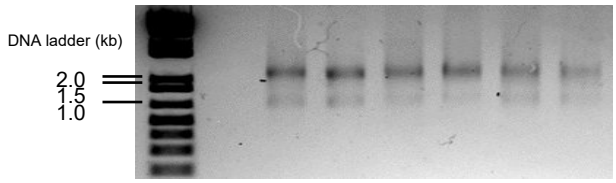
The RandomhexRT primer was purified by high-performance liquid chromatography (HPLC), while the other primers were purified using PAGE. All the primers were synthesized by Sangon Biotech.

Supplementary Table 1. Other primers

RandomhexRT	GTTCAGACGTGTGCTCTTCCGATCTNNNNNN
P5	AATGATACGGCGACCACCGAGATCTACACACACTCTTTCCCTACACGACGCTCTTCC
Index-1	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCC
Index-2	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCC
Index-5	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCC
Index-7	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCC
Index-4	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCC
Index-6	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCC
Index-8	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCC

Method

1. Prior to constructing the sequencing library, dilute all RNA samples to 25 ng/μL. Next, take 5 μL of several random samples to run on a 1% agarose gel in TBE buffer to check the RNA quality.
   Here is an example:



   For a total RNA sample with good quality, you will see two main bands, the upper 26S rRNA band and the lower 18S rRNA band, without obvious signs of degradation. If RNA is degraded, you will see bands or smears under 1.0 kb on the DNA ladder.

2. Begin library construction by performing **first strand reverse transcription** (RT1). This step barcodes the samples and needs to be done in 96-well PCR plate. Perform the experiment following the steps below.
   a. In a 96-well PCR plate, add the following reagents to each well:
      - Total RNA (25 ng/μL): 2 μL
      - Barcoding primer (2.5 μM): 2 μL
      - dNTP mix (dilute to 2.5 mM of each): 2 μL
   b. Spin down the 96-well plate and incubate the mixture at 65℃ for 5 min, and then cool down on ice for 2 min.
   c. Mix the following reagents and add 5 μL of the mix to each well of the 96-well plate:
      - First Strand Buffer: 220 μL
      - DTT (0.1 M): 50 μL
      - RNaseOUT: 30 μL
      - SuperScript™ III: 10 μL
      - H_2O: 200 μL
   d. Spin down the 96-well plate and incubate the plate at 50℃ for 90 min; then 70℃ for another 20 min.
      Checkpoint*: keep the products at 4℃ for several hours, or -80℃ for several months.*

3. Pool half of the RT1 products for each sample into Eppendorf tubes while keeping everything on ice.
   We recommend pooling ~50 samples to one sequencing library (tube). After pooling, purify the pooled library with 1.2X AMPure XP beads following the original CEL-Seq2 protocol [9], hereinafter follow the same protocol for beads purification). Elute the beads with 25 μL H_2O for each library. This produces 25 μL RT1 product for each pooled library.

4. **Exonuclease I digestion**: this step is to remove residual primers from the first reverse transcription step.

For each library, mix the following reagents on ice:

- RT1 product: 25 μL
- 10X digestion buffer: 3 μL
- Exonuclease I: 2 μL

Then briefly vortex the mixture and incubate it at 37℃ for 30 min. Purify the products with 1.2X AMPure XP beads. Elute the beads with 25 μL H_2O for each library.

5. Perform the **second strand cDNA synthesis** with the following steps.
    a. For each library, mix the following reagents on ice:
        - Elution from step 4: 22.2 μL
        - 5X second strand reaction buffer: 6 μL
        - dNTP mix (10 mM): 0.6 μL
        - *E. coli* DNA ligase (10 u/μL): 0.2 μL
        - DNA polymerase I (10 u/μL): 0.8 μL
        - Ribonuclease H (2 u/μL): 0.2 μL
    b. Then briefly vortex the mixture and incubate it at 16℃ for 2 hours with lid open and then at 65℃ for 20 min with lid heated at > 80℃.
    Checkpoint*: keep the products at 4℃ for several hours, or -80℃ for several months.*
    c. Purify the products with 1.2X AMPure XP beads. Elute the beads with 5 μL H_2O for each library.

    Quality Control: Take 1 μL purified product to measure the concentration using the Qubit dsDNA HS (High Sensitivity) Assay Kit. Here is an example of the results of 6 libraries in one batch:

	Lib1	Lib 2	Lib 3	Lib 4	Lib 5	Lib 6
Concentration (ng/μL)	2.16	2.14	2.71	2.52	2.38	2.60

    We are not sure if the concentration reflects the real concentration of dsDNA, but the measured concentration positively correlated with the RNA yield in the following In vitro transcription step. We recommend a minimum concentration of 1 ng/μL for a library pooled with ~50 samples to proceed to the next step.

6. ***In vitro*** **transcription** (IVT). Use HiScribe™ T7 High Yield RNA Synthesis Kit to perform IVT with the following steps.

    a. For each library mix the following reagents at room temperature:
        - Product from step 5: 4 μL
        - NTPs: 1 μL of each (4 μL total)
        - 10X IVT buffer: 1 μL
        - T7 enzyme: 1 μL

Briefly vortex the mixture and incubate it at 37℃ overnight in a PCR machine.

b. Purify RNA using 1.8X RNAClean XP beads. Elute the beads with 30 μL H_2O for each library.

Quality Control: measure the product concentration by NanoDrop. Here is an example of the results of 6 libraries in one batch:

	Lib1	Lib 2	Lib 3	Lib 4	Lib 5	Lib 6
Concentration (ng/μL)	572	660	627	529	532	582

For most libraries in our hands, the concentration of IVT products were more than 300 ng/μL. We recommend a minimal concentration of 100 ng/μL for a library pooled with ~50 samples to proceed to the next step.

c. Diute all IVT products to 300 ng/μL.

7. **Fragmentation**: this step is to fragment the IVT RNA products.
   a. For each library mix the following reagents on ice:
      - IVT RNA products: 4 μL (1.2 μg in total. If IVT RNA products concentration is less than 300 ng/μL, increase the volume to make the total input to be ~1.2 μg)
      - H_2O: 5 μL (If IVT RNA products concentration is less than 300 ng/μL, decrease the volume to make the total volume of IVT RNA products and H_2O be 9 μL)
      - 10X Fragmentation Buffer: 1 μL
   b. Briefly vortex the mixture and incubate at 70℃ for 2 min.
   c. Add 1 μL of the Stop Solution and immediately proceed to purify the products with ice cold 1.8X RNA Clean XP beads. Elute the beads with 20 μL H_2O for each library.

Quality Control 1: measure the product concentration by NanoDrop. Here is an example of the results of 6 libraries in one batch:

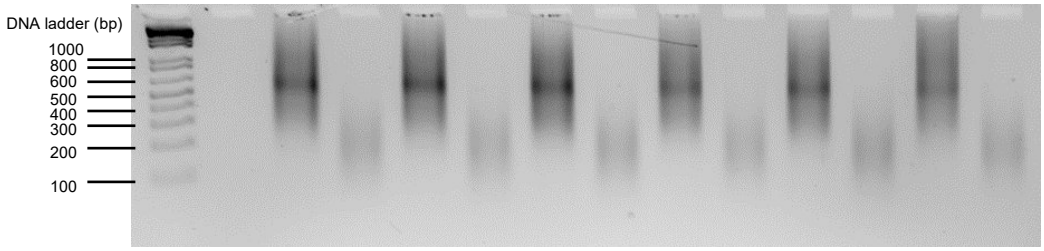
	Lib1	Lib 2	Lib 3	Lib 4	Lib 5	Lib 6
Concentration (ng/μL)	47.8	41.8	46.8	46.2	44.4	39.0

The concentration of fragmented products was usually more than 30 ng/μL in our hands. The recovery rate is >50% and ~70% for most cases.

d. Dilute all samples to 25 ng/μL.

Quality Control 2: Run a 1.2% TBE gel using 1.5 μL diluted IVT products or 5 μL diluted fragmentation product to check RNA size. Here is a gel image of 6 IVT products (left to right) before (larger-size band) and after (smaller-

size band) fragmentation.



The size of IVT and Fragmentation products were very stable in our libraries. The main band of IVT product is at the position between 500 bp to 600 bp of the DNA ladder while the Fragmentation product is at the position between 100 bp to 200 bp of DNA ladder. A smaller IVT product size indicates an inefficient RT1 reaction. A larger Fragmentation product size indicates inefficient fragmentation, while smaller Fragmentation product size indicates over fragmentation. (Please be aware that the DNA ladder only provides a reference for the relative fragment size; it does not indicate the actual sizes of RNA fragments.)

8. **The second reverse transcription** (RT2): this step is to generate cDNA from the fragmented RNA and add adaptor sequence to the cDNA product.
    a. For each library mix the following reagents on ice:
        - Fragmented RNA (25 ng/μL): 3 μL
        - RandomhexRT primer (10 μM): 2 μL
        - dNTP mix (10 mM of each): 1 μL
        - H_2O: 7 μL
    b. briefly vortex the mixture and incubate it at 65℃ for 5 min and then cool on ice for 2 min.
    c. add the following reagents to each library reaction on ice:
        - First Strand Buffer: 4 μL
        - DTT (0.1 M): 2 μL
        - RNaseOUT: 0.5 μL
        - SuperScript™ III: 0.5 μL
    d. briefly vortex the mixture and incubate it with the following temperature in a PCR machine:
        - 25 ℃ 10 min,
        - 50 ℃ 60 min,
        - 70 ℃ 15min.
    e. Purify the products with 1.2X AMPure XP beads. Elute the beads with 20 μL H_2O for each library.

9. **PCR amplification**: this is the final step for WPS library construction and produces the final library for sequencing.
    a. For each library mix the following reagents on ice:
        - RT2 product: 10 μL
        - H_2O: 8 μL

- Index primer primer (10 μM): 1 μL
- P5 primer (10 μM): 1 μL
- Kapa HIFI 2X mix: 20 μL

b. briefly vortex the mixture, then do PCR using the following cycling conditions:
   - 98 ℃ 2 min,
   - 12 cycles of
     1) [98 ℃ 10 s
     2) 60 ℃ 30 s
     3) 72 ℃ 30 s],
   - 72 ℃ 5 min,
   - Hold at 4 ℃.

c. Use 1X AMPure XP beads to purify the PCR products. Elute the beads with 25 μL H_2O for each library.
d. Use 1X AMPure XP beads to purify the 25 μL eluent from step c. Elute the beads with 25 μL H_2O for each library.

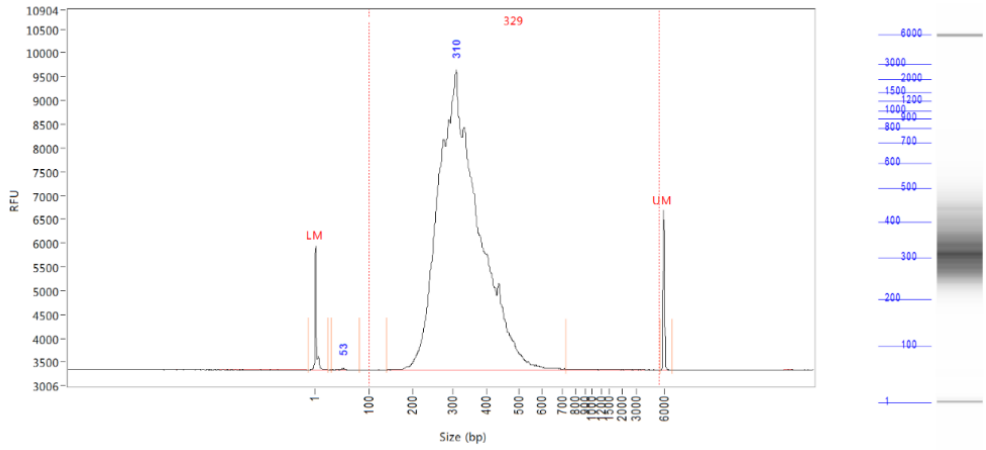
Quality Control: measure the library concentration by Qubit dsDNA HS (High Sensitivity) Assay Kit. Here is an example of the results of 6 libraries in one batch:

	Lib1	Lib 2	Lib 3	Lib 4	Lib 5	Lib 6
Concentration (ng/μL)	11.6	12.7	13.4	12.3	12.6	12.4

The concentration of libraries is usually ~10 ng/μL. >5 ng/μL is acceptable.

10. <u>Pre-sequencing quality control of final libraries</u>
    This step is to check the size distribution of the final library. We use Qubit to quantify concentrations of libraries and use one of the following two methods to check the size distribution of each library.

    Method 1: Fragment analyzer
    Mix 1 μL library and 2 μL H_2O and run Fragment analyzer with the 3 μL solution. Here is an example of fragment analyzer result of a good library:



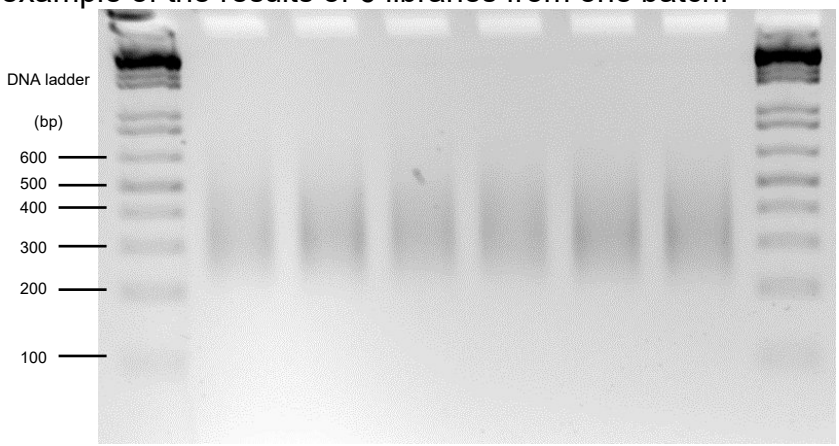
Peak	Size (bp)	Conc. (ng/uL)	From (bp)	To (bp)	Avg. Size (bp)	CV%	RFU	Corr. Peak Area
1	1 (LM)	0.0152	0	26	2	172.70	2609	16.540
2	53	0.0054	31	84	50	8.80	36	0.490
3	310	8.2779	140	728	327	19.99	6304	748.240
4	6000 (UM)	0.0091	5716	6873	5995	1.30	3356	9.871
	TIC:	8.2834	ng/uL					
	TIM:	41.822	nmole/L					
	Total Conc.:	8.2992	ng/uL					

Smear Analysis 100 bp to 5500 bp 8.2937 ng/ul 99.9 %Total 41.408 nmole/L 329 Avg. Size (b.p.) 27.57 %CV

The peak size is expected to be ~300 bp with peak expansion from 200 bp to ~500 bp. Any obvious shift from this range indicates problems in previous steps (e.g., RT1 and fragmentation) and may cause trouble in the sequencing step.

Method 2: Gel electrophoresis

We found that it is robust enough to simply run ~30 ng or more of the final library on a 1.2% TBE gel for quality control of the fragment size. This is more cost efficient and faster than the Fragment analyzer that requires a special facility. Here is an example of the results of 6 libraries from one batch:



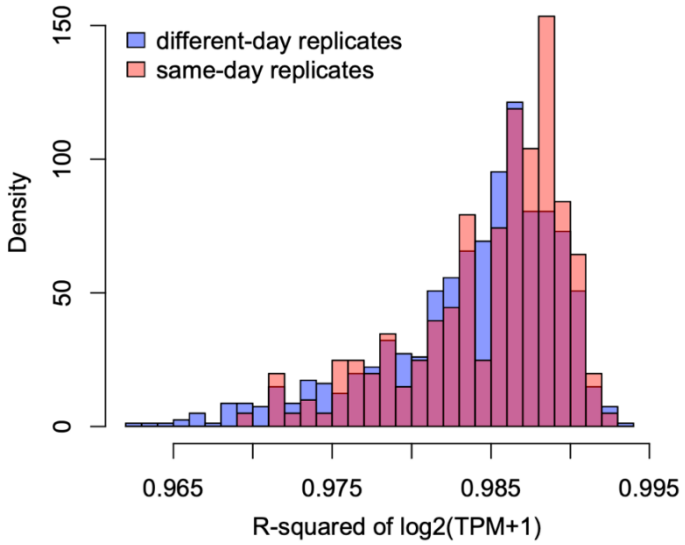
We expect the main band spread to be between 200-500 bp with a peak at ~300 bp. Any obvious shift from this range needs to be further diagnosed by fragment analyzer or resolved by remaking the library.

Section IV: Data analysis

Please refer to https://github.com/XuhangLi/WPS for a full tutorial of WPS data analysis.

Notes on the use of same-day vector controls

As discussed in the previous sections, standard WPS protocol prepares two independent cultures of vector control in each experiment, which we refer to as same-day replicates. This is in principle different than the stringent biological replicates that are done in different days. Interestingly, we found that the level of gene expression correlation between same-day replicates closely aligned with that between different-day replicates (**Supplementary Fig. 14**). Therefore, we used both same-day and different-day replicates in our DE analysis to increase power. We recommend that users inspect their own data to determine



Supplementary Fig. 14: Gene expression correlation between vector control samples of same-day or different-day replicates. X-axis shows the correlation level (r^2) calculated using log2(TPM+1) of detected genes between all pairs of same-day replicates (red) or different-day replicates (blue) from the metabolic WPS dataset.

If both replicates should be included. Specifically, please (1) confirm that a similar closely aligned variance level is observed, and (2) test DE analysis using either both types of replicates or only the different-day replicates and check the differences in the results. Based on our experience, DE analysis using both replicates in fact gives more conservative results (less DEGs) in many conditions.

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