Supplemental Materials and Methods

Proteomic sample preparation and analysis

Two distinct *Buchnera* life stages were collected to determine if *Buchnera* gene regulation occurs at the protein level. As noted in the main text *Buchnera* cells were collected for the embryo sample by dissection and for the maternal bacteriocytes by filtration and differential centrifugation of 4th instar *A*. *pisum* (str. LSR1). Although whole body preparations that were filtered contained embryos along with maternal bacteriocytes, *Buchnera* from maternal bacteriocytes dominate because embryos are sticky and are largely removed during early filtration steps, and because most *Buchnera* cells in 4th instar aphids are in maternal bacteriocytes and not in embryo tissues; embryos are relatively small in 4th instar aphids (A.K.H. personal observation). Moreover, using data from a previous study using Label free quantification (LFQ) (Poliakov *et al.,* 2011) we found that *Buchnera* protein expression from whole body samples was nearly identical to expression from dissected bacteriocyte samples (Pearson correlation coefficient = 0.974; $P < 0.001$, N = 397 proteins). The whole body sample preparation from Poliakov *et al.* (2011) is also very similar to *Buchnera* protein expression from the whole body, filtered sample preparation of our study (Pearson correlation coefficient = 0.977; $P < 0.001$, N = 301 proteins), but less similar to our dissected embryo protein preparation (Pearson correlation coefficient = 0.947; $P < 0.001$, N = 301 proteins). In turn we will refer to our second sample preparation as “bacteriocyte” throughout the manuscript, since

this sample preparation primarily contains *Buchnera* cells from maternal bacteriocytes.

To determine changes in *Buchnera* protein expression between embryo and bacteriocytes, Label Free Quantification was conducted by WM Keck Foundation Biotechnology Resource Laboratory at Yale University. A proportional amount of RIPA buffer was added to each sample relative to initial sample volume, e.g. 200 µL of RIPA was added to the bacteriocytes sample and 100 µL was added to the embryo sample (RIPA- 0.15 M NaCl, 10 mM TRIS, 0.1% SDS, 0.1% TritonX 100, 0.01% deoxycholate and 5 mM EDTA). Samples were sonicated three times for 20 sec each time on ice with 0.5 sec pulses. This protein preparation step was especially important for the purification of embryo tissues since embryos are very sticky. Samples were subsequently spun at 14,000 rpm to pellet debris. A chloroform / methanol precipitation was followed by a Lys C /Tryptic digestion. Samples were diluted and 0.25 µg was loaded on the column and run in triplicate. LC-MS/MS on the LTQ Orbitrap ELITE was then conducted. The LTQ Orbitrap is equipped with a Waters nanoAcquity UPLC system, and uses a Waters Symmetry® C18 180 µm x 20 mm trap column and a 1.7 µm, 75 µm x 250 mm nanoAcquity™ UPLC™ column (35ºC) for peptide separation. Trapping is done at 5 µL/min, 99% Buffer A (100% water, 0.1% formic acid) for 3 min. Peptide separation was performed with a linear gradient over 200 minutes at a flow rate of 300 nL/min.

The WM Keck Foundation Biotechnology Resource Laboratory at Yale University also performed the subsequent processing and analysis of the

proteomic data. Briefly, the Mascot software suite was used to identify peptide matches in a database comprised of proteins from the sequenced bacterial and insect genomes of the pea aphid: *Buchnera aphidicola* str. 5A (PRJNA59285) including pTrp and pLeu from *Buchnera aphidicola* str. APS (PRJNA57805), and *Acyrthosiphon pisum* str. LSR1 v2.0 chromosomal and mitochondrial proteins (PRJNA13657 and PRJNA29489). Confidence level was set to 95% within the MASCOT search engine for protein hits based on randomness. For data analysis, feature extraction, chromatographic/spectral alignment, data filtering, and statistical analysis were performed using Nonlinear Dynamics Progenesis LC-MS software (www.nonlinear.com); Progenesis LCMS (Nonlinear Dynamics, LLC). First, the raw data files were imported into the program. A sample run was chosen as a reference (usually at or near the middle of all runs in a set), and all other runs were automatically aligned to that run in order to minimize retention time (RT) variability between runs. No adjustments are necessary in the m/z dimension due to the high mass accuracy of the spectrometer (typically < 3ppm). All runs were selected for detection with an automatic detection limit. Retention time range of 0–8 and 180–200 minutes were filtered out. Charge left of +1 to +7, mascot peptide scores of < 26, and peptide masses < 750 were deleted. A normalization factor was then calculated for each run to account for differences in sample load between injections. The experimental design was set up to group multiple injections from each run. Because the two *Buchnera* samples contained a mixture of both insect and bacterial proteins, *Buchnera* abundances were normalized to a constitutively expressed *Buchnera* protein, 50S ribosomal

subunit protein RplN (BUAP5A_507). This protein was determined as an ideal normalizing housekeeping protein for two reasons, first after normalization this *Buchnera* protein was not statistically different compared to the vast majority of other constitutively expressed proteins, and second it contained ≥ 3 peptide counts per sample. Additionally, *rplN* appeared ideal for normalizing mRNA transcripts as well as proteins (see below for qRT-PCR). Raw and normalized abundances, maximum fold change, and ANOVA values for each feature in the data set was calculated by the Nonlinear Dynamics Progenesis LC-MS algorithm, and protein identifies were imported from MASCOT. Statistical analysis and criteria closely followed Ahmed et al. (2013), Babaei et al. (2013), Bostanci et al. (2013), and Brownridge et al. (2013), which used a similar Progenesis LCMS statistical analysis pipeline (Nonlinear Dynamics, LLC.). Specifically, to identify differentially expressed proteins between treatments the *P*-value for the one-way ANOVA was calculated from the sum of the normalized abundance across all runs similar to Babaei et al. (2013). In addition to the *P*-value criterion, a conservative threshold of 1.5-fold was chosen for biological significance similar to Blagoev et al. (2004).

Since not all *Buchnera* proteins were detected in LFQ in this study and a previous study (Poliakov *et al.,* 2011), we conducted another proteomics method (MudPIT) for protein identity. MudPIT was conducted on another biological replicate of the whole body-bacteriocytes preparation as detailed above for 4th instar *A*. *pisum* (str. LSR1). The sample was digested in 2 M urea, 0.1 M NH_4HCO_3 after reduction and alkylation of the cysteine to carbamidomethylated

cystiene. The digest was then separated by strong cation exchange off line with 20 fractions collected; each fraction was then analyzed by LC-MS/MS analysis on the LTQ Orbitrap with peptide separation on a Waters nanoACQUITY (75 µm x 250 mm eluted at 300nL/min.). Mascot distiller and the Mascot search algorithm were used for database searching against the same database detailed above for LFQ analyses.

RT-qPCR

We compared *Buchnera*-LSR1 mRNA gene expression for three aphid tissue types (e.g., young embryo (YE), old embryo (OE), and maternal bacteriocyte (BAC)). These tissues harbor *Buchnera* representing different life stages and can be distinguished based on tissue size and morphology (Braendle *et al.,* 2003; Koga *et al.,* 2012). We define YE as stage 8 or lower, while OE are stage 10 or higher. As above, YE, OE and BAC samples were rapidly dissected out of 4th instar *A. pisum* (str. LSR1), pooled in RNA Bacterial Protect (Qiagen) and stored at -80ºC before RNA extraction, DNAase treatment and cDNA synthesis (as described in main text).

Manufacturer's recommended protocols for SYBR Fast Universal qPCR reagents (KAPA Biosystems, Massachusetts) were followed for reaction chemistry and 2-step cycling conditions in a Mastercycler Epgradient Realplex^2 (Eppendorf, Hamburg) thermocycler. Target and housekeeping amplicons were cloned into the pGEM-T vector (Promega, Wisoconsin) and used to produce standard curves (oligonucleotide primers listed in Supplemental Table S1).

Appropriate standards were run on every plate using a 10-fold dilution series. Initial validation experiments demonstrated that *rplN* (BUAP5A_507) was constitutively expressed in all three sample types and showed no significant difference relative to other putative housekeeping genes (e.g., *rpsU*, *rrf*, *rpsQ*, *rplT*, and *yheL*) (data not shown). The standard curve method for relative quantification (Bookout *et al.,* 2006) was used with plasmids to compute the normalized *Buchnera* value for each sample (i.e., normalized to the *Buchnera* housekeeping gene *rplN*). As mentioned in the main text statistical analyses were performed with CLC Genomics Workbench; Kal's Z-test (Kal *et al.,* 1999), and multiple comparisons with a false discovery rate criterion of α =0.05 or less.

Reanalysis of life stage microarray data

Raw data from Bermingham *et al.* (2009) were retrieved from the ArrayExpress repository (E-MEXP-2057; EMBL-EBI). Using the Bioconductor package Marray in R, probe expression levels *within* individual microarrays were first median corrected for background fluorescence and then normalized independently by the Loess method. Probe expression values *between* microarrays were then normalized by the Scale method yielding relative gene expression values (Supplemental Fig. 4; M = log2 (T/C), where T is the fluorescent signal from the treatment and C is control). A custom Perl script was written to calculate the average expression values of all probes corresponding to individual *Buchnera* genes, excluding probes identified by the GenePix software as 'bad', 'absent' or 'not found'. Statistics were carried out on mean expression values in CLC Genomics Workbench. Pair-wise comparisons of individual genes between an

embryo treatment and the bacteriocyte control were conducted with *t*-tests and gene group comparisons were conducted with global ANOVA. A FDR criterion of $P \leq 0.05$ was conducted for both pairwise and group analyses.

Supplemental Results

We applied the criteria of Bermingham et al. (2009) to determine significantly up or down regulated genes: up-regulated genes are ≥ 1.5-fold higher and down-regulated genes are ≤ 0.66-fold lower, and have a significant difference of ($P < 0.05$) in a global ANOVA and at least one group comparison. In contrast to the 140 genes and tRNAs previously identified as being significantly expressed, only 9 genes and 2 tRNAs in our reanalysis were significant based on these criteria. When a False Discovery Rate (FDR) of $P \leq 0.05$ was considered none of these genes were significantly regulated (Supplemental Figure S4).

Supplemental References

Ahmed S, Maratha A, Butt AQ, Shevlin E, Miggin SM. (2013) TRIF-mediated TLR3 and TLR4 signaling is negatively regulated by ADAM15. *J Immunol* **190:** 2217–2228.

Babaei F, Ramalingam R, Tavendale A, Liang Y, Yan LS, Ajuh P *et al*. (2013) Novel blood collection method allows plasma proteome analysis from single zebrafish. *J Proteome Res* **12:** 1580–1590.

Blagoev B, Ong S, Kratchmarova I, Mann M. (2004) Temporal analysis of phosphotyrosine-dependent signaling networks by quantitative proteomics. *Nature Biotechnology* **22:** 1139–1145.

Bostanci N, Ramberg P, Wahlander Å, Grossman J, Jönsson D, Barnes VM *et al*. (2013) Label-free quantitative proteomics reveals differentially regulated proteins in experimental gingivitis. *J Proteome Res* **12:** 657–678.

Brownridge P, Lawless C, Payapilly AB, Lanthaler K, Holman SW, Harman VM *et al.* (2013) Quantitative analysis of chaperone network throughput in budding yeast. *Proteomics* **13:** 1276–1291.

Supplemental Figure Legends

Figure S1. Lengths of predicted *Buchnera* UTRs are highly correlated. Lengths of 5' and 3' UTRs from individual genes shared by each genome pair are plotted in the bottom and the top of the matrix respectively. Pearson correlation coefficients for each genome comparison and density ellipse (red) are shown. Spearman's Nonparametric test was performed; * = $P < 0.05$, ** = $P < 0.0001$.

Figure S2. Genes flanking intergenic sRNAs have a range of protein expression profiles. Of the 25 discreet intergenic sRNAs identified, the genes flanking 14 of them were tested by qRT-PCR and show no difference in mRNA expression was observed between three distinct life stages (see Supplemental Table S4). However, protein expression measured by LFQ and MudPIT protein detection was found to vary, suggesting a potential for post-transcriptional regulation by sRNAs or other mechanisms.

Figure S3. *Buchnera* proteomic profiles are similar between techniques and studies. Venn diagram of *Buchnera*-LSR1 proteins detected in this study by LFQ and MudPIT analyses compared with a summary of independent LFQ analyses described by Poliakov et al. (2011). A considerable fraction of coding sequences present in the *Buchnera*-LSR1 genome have never been detected as proteins.

Figure S4. *Buchnera* gene expression in early, intermediate and late embryos is not significantly different than that in bacteriocytes. Box plots in (**a**-**c**) show the effect of quality processing of relative gene expression values (M) for individual *Buchnera* probes from the microarray data in Bermingham et al. (2009). Raw microarray data (**a**) were subjected to *within* slide normalization by median background subtraction and the Loess method (**b**), followed by *between* slide normalization using the Scale method (**c**). Volcano plots in (**d-f**) show differences in relative gene expression values (M) between (**d**) early, (**e**) intermediate and (**f**) late embryos *vs.* bacteriocytes plotted against their raw (blue) and FDR corrected (red) *P* values. Vertical dotted lines indicate fold change thresholds of ≥ 1.5-fold up and ≤ 0.66-fold down regulation. Horizontal dotted lines indicate the *P* value cutoff of 0.05.

Supplemental Tables (see included spreadsheets)

Table S1. *Buchnera* proteins determined by LFQ to be differentially expressed in *A. pisum* Embryos compared to Bacteriocytes

Table S2. Identification of *Buchnera* proteins expressed in *A. pisum* maternal bacteriocytes detected by MudPIT

Table S3. Statistical comparisons of RNA expression levels of sixty *Buchnera*-LSR1 genes during distinct life stages by qRT-PCR

Table S4. Expressed and structurally conserved untranslated regions (UTRs) in *Buchnera*

Table S5. Expression and structural conservation of sRNAs located in *Buchnera* intergenic spacers

Table S6. Expression and structural conservation of asRNAs in *Buchnera*

Table S7. Primers used for quantitative RT-PCR

Figure S1

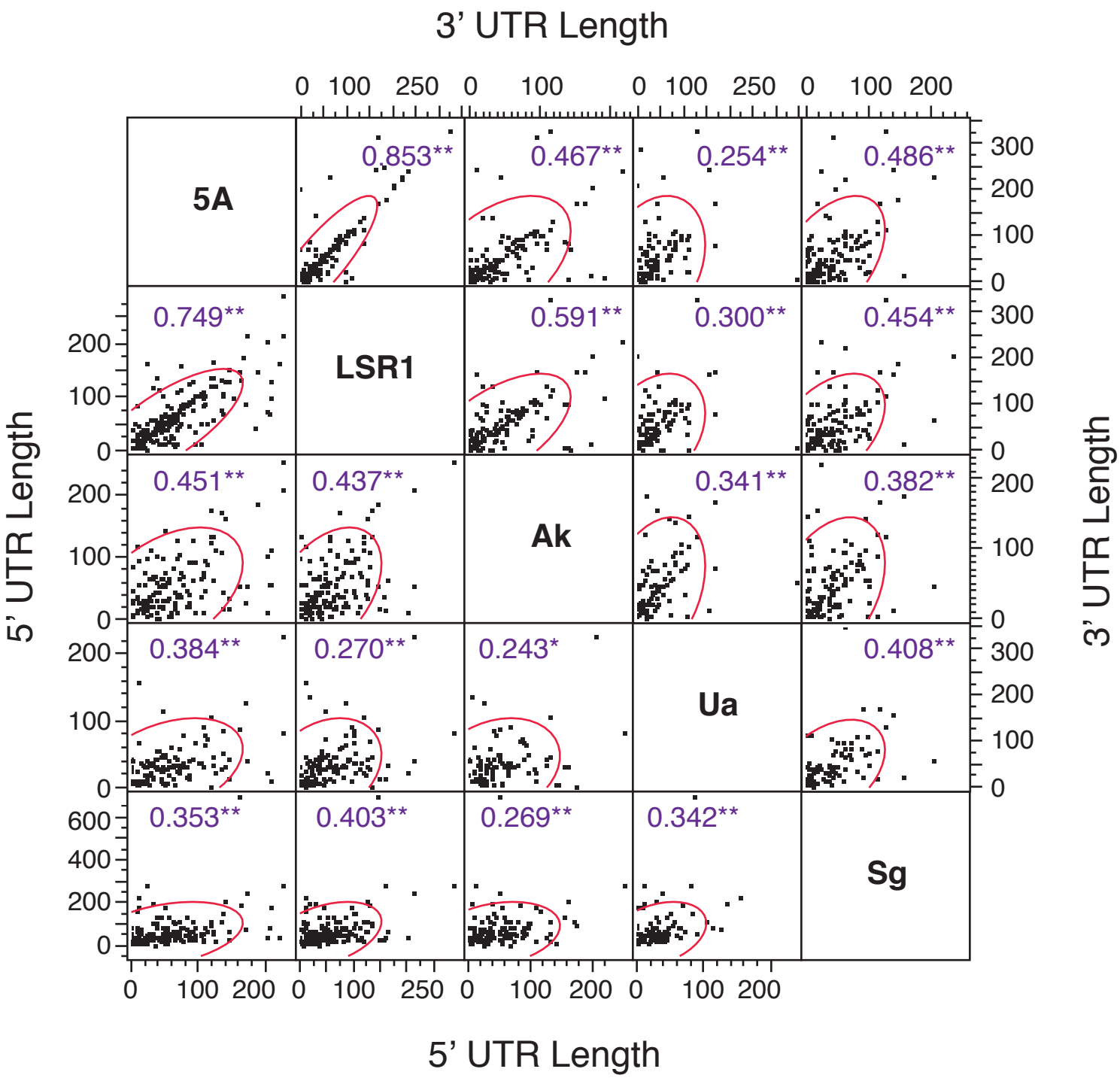

3’ UTR Length
5’ UTR Length
3’ UTR Length
5’ UTR Length
5A
LSR1
Ak
Ua
Sg
0.853**
0.467**
0.254**
0.486**
0.749**
0.591**
0.300**
0.454**
0.451**
0.437**
0.341**
0.382**
0.384**
0.270**
0.243*
0.408**
0.353**
0.403**
0.269**
0.342**

Figure S2

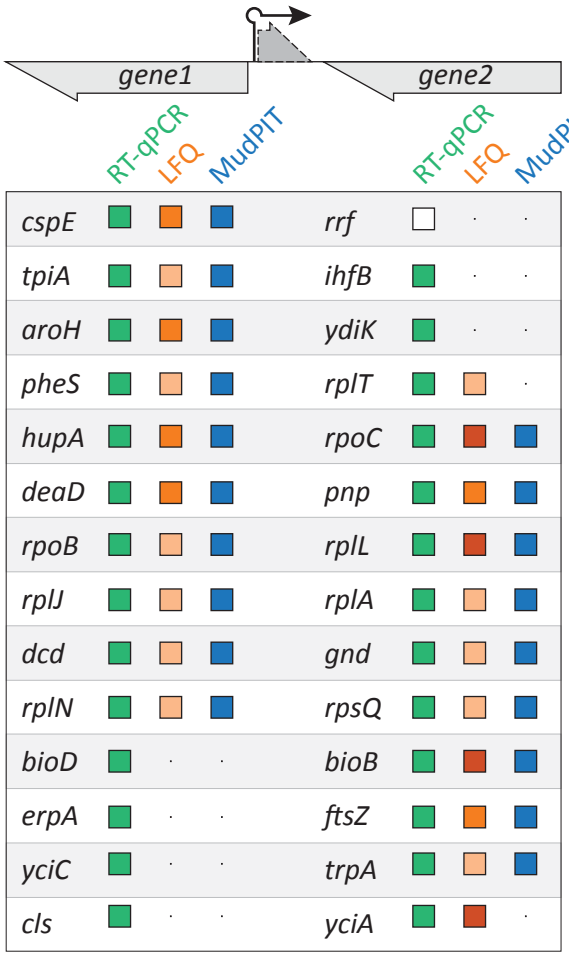

gene1
gene2
RT-qPCR
LFQ
MudPIT
RT-qPCR
LFQ
MudPIT
cspE
tpiA
aroH
pheS
hupA
deaD
rpoB
rplJ
dcd
rplN
bioD
erpA
yciC
cls
rrf
ihfB
ydiK
rplT
rpoC
pnp
rplL
rplA
gnd
rpsQ
bioB
ftsZ
trpA
yciA
RT-qPCR
early & late embryos, bacteriocytes
n.m.
n.s.
LFQ
ovaries/ bacteriocytes
-1
-0.5
0.5
1
n.d.
q1
q2
q3
q4
MudPIT
bacteriocytes
n.d.
detected

Figure S3

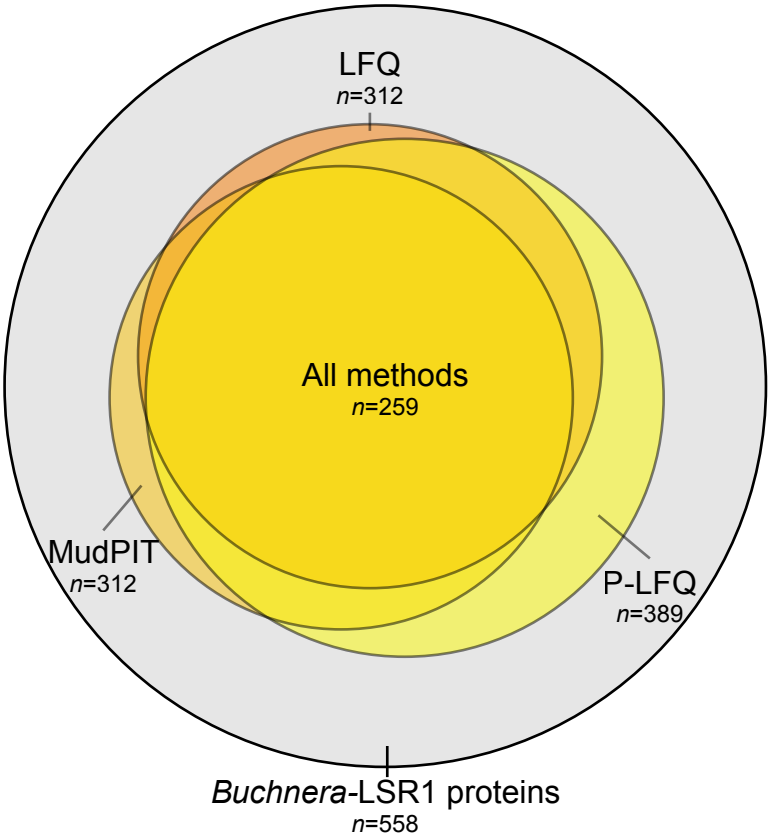
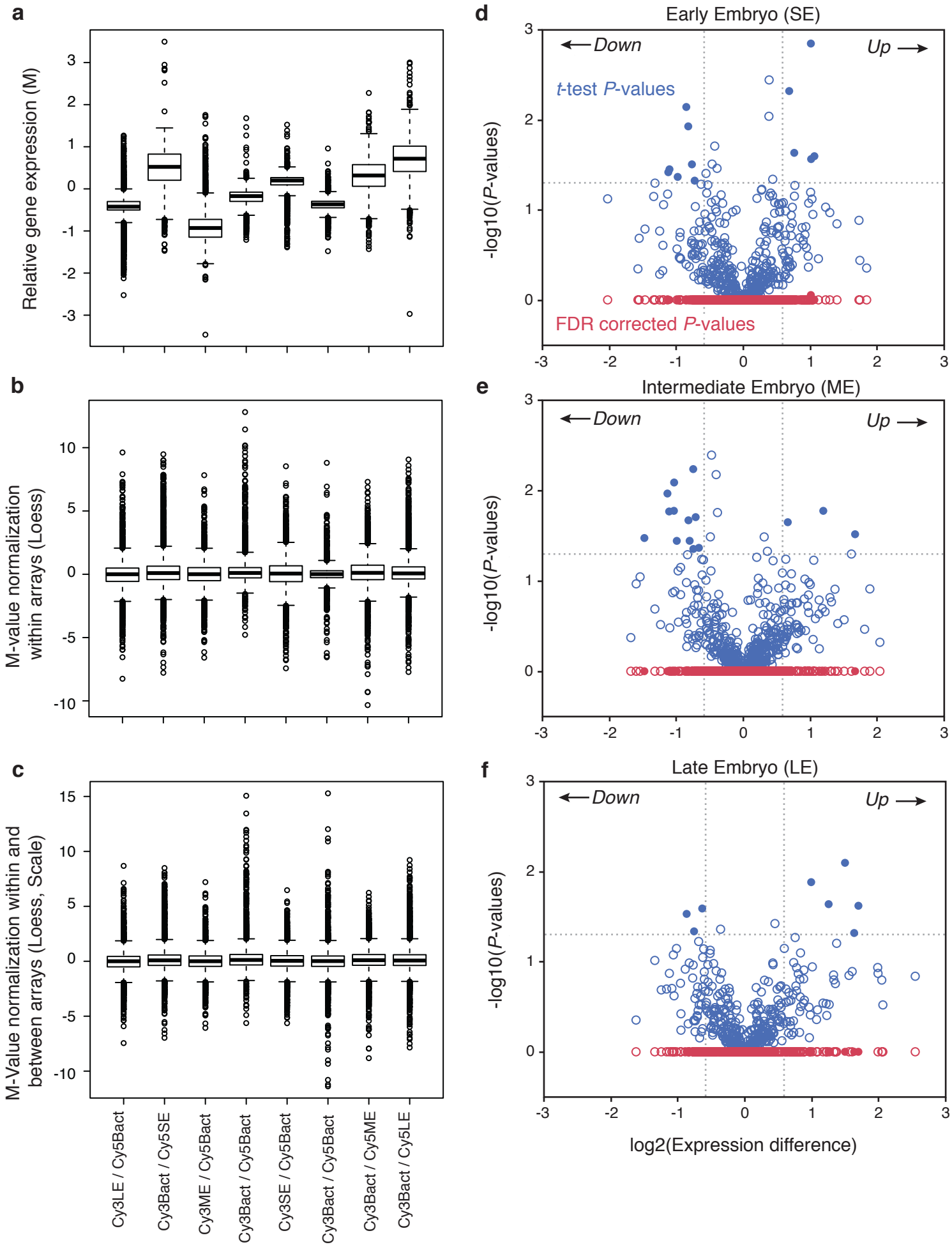

LFQ
n=312
All methods
n=259
MudPIT
n=312
P-LFQ
n=389
Buchnera-LSR1 proteins
n=558

Figure S4


a
Relative gene expression (M)
b
M-value normalization within arrays (Loess)
c
M-Value normalization within and between arrays (Loess, Scale)
Cy3LE / Cy5Bact
Cy3Bact / Cy5SE
Cy3ME / Cy5Bact
Cy3Bact / Cy5Bact
Cy3SE / Cy5Bact
Cy3Bact / Cy5Bact
Cy3Bact / Cy5ME
Cy3Bact / Cy5LE
d
Early Embryo (SE)
e
Intermediate Embryo (ME)
f
Late Embryo (LE)
Down
Up
t-test P-values
FDR corrected P-values
-log10(P-values)
log2(Expression difference)