Quantitative Single-Embryo Profile of *Drosophila* Genome Activation and the Dorsal–Ventral Patterning Network

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ABSTRACT During embryonic development of *Drosophila melanogaster*, the maternal-to-zygotic transition (MZT) marks a significant and rapid turning point when zygotic transcription begins and control of development is transferred from maternally deposited transcripts. Characterizing the sequential activation of the genome during the MZT requires precise timing and a sensitive assay to measure changes in expression. We utilized the NanoString nCounter instrument, which directly counts messenger RNA transcripts without reverse transcription or amplification, to study >70 genes expressed along the dorsal–ventral (DV) axis of early *Drosophila* embryos, dividing the MZT into 10 time points. Transcripts were quantified for every gene studied at all time points, providing the first dataset of absolute numbers of transcripts during *Drosophila* development. We found that gene expression changes quickly during the MZT, with early nuclear cycle 14 (NC14) the most dynamic time for the embryo. *twist* is one of the most abundant genes in the entire embryo and we use mutants to quantitatively demonstrate how it cooperates with Dorsal to activate transcription and is responsible for some of the rapid changes in transcription observed during early NC14. We also uncovered elements within the gene regulatory network that maintain precise transcript levels for sets of genes that are spatiotemporally cotranscribed within the presumptive mesoderm or dorsal ectoderm. Using these new data, we show that a fine-scale, quantitative analysis of temporal gene expression can provide new insights into developmental biology by uncovering trends in gene networks, including coregulation of target genes and specific temporal input by transcription factors.

KEYWORDS *Drosophila melanogaster*; gene expression; transcription; dorsal–ventral patterning; maternal-to-zygotic transition; NanoString nCounter

THE maternal-to-zygotic transition (MZT) is a key step in animal embryonic development, when maternally deposited transcripts are degraded in the embryo, and the embryonic genome is first activated. In *Drosophila melanogaster*, the MZT takes place within the first 3 hr of development, during the late syncytial nuclear divisions and ending at the cellular blastoderm stage with gastrulation (Foe and Alberts 1983; Pritchard and Schubiger 1996; Tadros and Lipshitz 2009). Gene expression during the MZT is highly dynamic, with patterns of zygotic genes first being established and changing between and within nuclear cycles (Stathopoulos and Levine 2005; Reeves *et al.* 2012). It is

clear, therefore, that each syncytial nuclear cycle can be treated as a single, or even multiple developmental time points. A few recent RNA-sequencing (RNA-seq)-based studies have in fact divided embryonic development into time points based on syncytial nuclear divisions for this very reason (Lott *et al.* 2011; Ali-Murthy *et al.* 2013). In previous studies, however, the syncytial nuclear stage, especially nuclear cycles 10–14 (NC10–14), have been grouped together in a small number of developmental stages or time points (Bownes 1975; Bate and Martinez Arias 1993; Graveley *et al.* 2011). These pioneering studies provided the basis for studying embryonic development of *Drosophila*, and the modENCODE transcriptome provided a depth of sequencing data never before achieved for *Drosophila*. We choose, however, to focus on a fine time scale approach and fewer genes to provide a detailed analysis of a specific period in development.

The top-level network inputs appear to be more dynamic on the DV axis than on the anterior–posterior (AP) axis. An

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activator of AP transcription is maternally deposited *bicoid*, which is transported to the anterior pole and forms a concentration gradient. The nuclear concentration of Bicoid during the final five nuclear cycles remains mostly constant during each nuclear cycle, indicating that Bicoid itself activates transcription of AP genes at a constant rate through these nuclear cycles (Gregor *et al.* 2007). In contrast, the protein product of the maternal gene *dorsal*, found in a DV gradient, increases in concentration within nuclei during each of the final five nuclear cycles (Reeves *et al.* 2012). This increase in nuclear Dorsal concentration suggests that the DV network is activated differently at each nuclear cycle, both by Dorsal itself, and by a network of transcription factors that respond to different levels of Dorsal. The combination of the rapidly changing transcriptional landscape during the MZT, the increasing nuclear concentration of Dorsal on the DV axis, and the small number of studies that have examined embryogenesis at the single nuclear cycle level present an opportunity to use emerging technologies to provide additional insight into this gene patterning network.

In this study, we examine the MZT and gene expression dynamics of the DV network at 10 time points during *Drosophila* embryonic development between NC10 and gastrulation at a 10- to 15-min resolution. We utilize the NanoString nCounter instrument to directly detect and quantify 68 early embryonic genes from single embryos, and we calculate the absolute number of transcripts per embryo for every gene at every time point in the study. The NanoString system is able to precisely quantify transcripts across five orders of magnitude from a single embryo without the need to fragment, amplify, or reverse transcribe the RNA (Geiss *et al.* 2008). The direct detection of messenger RNA (mRNA) molecules minimizes steps between sample collection and data acquisition, reducing error, sample loss, or contamination. RNA-seq has been used in past studies of the *Drosophila* MZT to quantify the number of transcripts for a gene in the early embryo, and while these studies provide an abundance of data for all genes transcribed, the methods used have been shown to introduce bias in transcript count and read coverage that can hamper absolute quantification of transcripts (Hansen *et al.* 2010; Lott *et al.* 2011; Roberts *et al.* 2011; Ali-Murthy *et al.* 2013; Petkova *et al.* 2014).

Materials and Methods

Fly stocks

Embryo collection and live imaging were done on flies with a His2Av-RFP fusion [Bloomington *Drosophila* Stock Center (BDSC) 23650]. *twist*⁻ (*twi*) embryos were obtained using a *twi*¹/CyO stock (BDSC 2381). PCR for LacZ was done on all mutant embryos to confirm the absence of the balancer chromosome and the presence of homozygous *twi*⁻ mutant chromosomes.

Live imaging and embryo collection

Flies with the His2Av-RFP fusion were allowed to lay eggs for 4 hr at 25°. Individual embryos were hand dechorionated and mounted on a microscope slide using a modified version

of the hanging-drop method (Reed *et al.* 2009). Nuclear divisions were monitored using epifluorescence, and confocal images of individual embryos were captured when embryos reached a desired developmental stage (Figure 1, A and B). NC13 was broken into two stages based on number of minutes into interphase, with early NC13 at five minutes into interphase, and NC late 13 at 12 min into interphase. NC14 was divided into four stages, 14A, 14B, 14C, and 14D, with embryo stage determined by three criteria: time elapsed in interphase, nuclear elongation, and progression of cellularization. NC14A was staged at 10–15 min into interphase, with a 1:1 ratio of nuclear length to width, and before the start of cellularization. NC14B was staged at 25–30 min with a nuclear elongation ratio of 2:1 and cellularization progressed <33%. NC14C was staged at 40–45 min with a nuclear elongation ratio of 3:1 and cellularization progressed <66%. NC14D was staged at 55–60 min with a nuclear elongation ratio >3:1 and cellularization progressed >66%. Selected embryos were placed in 100 µl Trizol reagent and snap frozen in liquid nitrogen within 1 min of imaging and stored at –80°. Confocal images of collected embryos were analyzed and the precise nuclear cycle was determined by calculating nuclear density.

RNA extraction and NanoString analysis

Embryos of desired developmental stage were selected based on confocal image analysis, thawed, and crushed, and 900 µl Trizol reagent was added. Additionally, 1 µl of Affymetrix GeneChip PolyA RNA control was added at a dilution of 1:10,000. RNA was extracted from Trizol reagent according to the standard protocol, except an additional chloroform extraction and an additional 70% ethanol wash were performed to increase the purity of RNA for hybridization. Purified RNA was resuspended in 10 µl RNase-free dH₂O and 1 µl was analyzed on a NanoDrop 2000 UV-Vis Spectrophotometer to determine RNA purity and concentration. A total of 5 µl of RNA from a single embryo was hybridized with NanoString probes at 65° for 18 hr and transcripts were quantified on the NanoString Digital Analyzer using the high-sensitivity protocol and 1155 fields of view. Three single embryos were analyzed for each time point and the average transcript count was used after normalization with GeneChip PolyA RNA controls and NanoString positive controls. Any NanoString experiments with abnormally high or low RNA spike-in counts were excluded from final data analysis and additional embryos were used to generate data.

A NanoString bioinformatics team carried out probe design so that all probes had similar binding properties and bound to one single exon that covered as many isoforms as possible for each gene. NanoString specifications indicate that hybridization efficiencies may vary by up to twofold. After data were collected from the NanoString nCounter, background was removed by averaging three RNA negative runs on the nCounter, averaging the count for each probe, and subtracting probe-specific background from each gene. For 75 probes, the background count was in the single digits, with the background count of a single probe giving

Figure 1 Timing of maternal-to-zygotic transition in the syncytial blastoderm, experimental methods, and controls. (A) Diagram of the degradation of maternal transcripts and the accumulation of zygotic transcripts. Embryo age in minutes after egg laying and corresponding nuclear cycle are displayed. (B) Confocal time series of *Drosophila* embryos expressing Histone2Av-RFP fusion. Nuclear density is used to determine nuclear cycle for NCs 10–14, and nuclear elongation (expanded images) is used to stage embryos within NC 14. (C) Linear regression of RNA spike in controls (blue) and NanoString positive controls (red). The graph displays both absolute number of control molecules added and number counted per sample for four foreign RNA spike-ins added to embryonic RNA during extraction and positive controls added to the NanoString probe mix during manufacture. (D) The dynamic range of transcription varies over four orders of magnitude between the least abundant (*pnr*) and most abundant (*dhd*) gene in the code set, but still completely within the six log dynamic range detection limit of the NanoString instrument. Error bars represent confidence interval $P \leq 0.001$. In this and other figures, number of transcripts refers to counts measured from single embryos, done in triplicate and averaged. (E) The genes *bcd* and *sna* have previously been quantified in the embryo during a single time point or subset of time points within the time course covered by this study. Their expression profiles calculated using NanoString, as measured in number of transcripts per embryo, are plotted. Error bars represent confidence interval $P \leq 0.001$. (F and G) qPCR comparing the abundance of *bcd* (F) and *sna* (G) to spike-in RNA controls shows that the ratio between *bcd* and *sna* transcripts and the controls is highly similar to the ratio calculated using NanoString. Error bars represent SEM.

250 counts. This probe was deemed defective by the manufacturer and excluded from the study. Supplemental Material, Figure S1 shows the raw background counts for all probes.

Data availability

Table S1 lists function and general expression domain and timing of *Drosophila* genes included in the study. Table S2 lists probe sequences used for NanoString code set. Table S3 provides quantified counts for all *Drosophila* genes in the code set.

Results

Creation of a developmental time series

We selected nuclear cycle 10 through gastrulation as the extent of the time series in order to focus on the beginning of the syncytial blastoderm stage when maternal transcripts are abundant and zygotic transcription is beginning, until gastrulation, when zygotic transcription is robust and many signaling pathways are functioning (Figure 1A). We staged individual embryos at each time point using a transgenic line of flies carrying a histone-RFP fusion, using fluorescence to

visually inspect and capture an image of each embryo immediately before collection. Nuclear cycle was confirmed by calculating nuclear density using confocal images. Immediately after imaging, embryos were immersed in TRIzol and snap frozen in liquid nitrogen (Figure 1B).

Control mRNA spike-ins were added during extraction to determine NanoString efficiency and calculate absolute number of transcripts per embryo in a manner not biased by number of cells or other measures that rely on embryonic transcription (Loven *et al.* 2012). RNA was hybridized with NanoString probes according to standard protocols, and the RNA-Probe hybrid molecules were bound to slides using the nCounter Prep Station and counted using the nCounter Digital Analyzer. Raw counts were normalized using both NanoString positive controls added to the probe mix during synthesis and mRNA spike-in controls added during extraction.

Quantification of transcripts and dynamic range of transcription

To compute the absolute number of transcripts for genes included in the dataset, we calculated a linear regression

($R^2 = 0.966$) for the mRNA spike-ins comparing input to NanoString counts, and fit counts for all other genes to this regression line. Using this fit, we calculated a scaling factor of 232.84 ± 11.52 (confidence interval $P \leq 0.001$) between NanoString counts and number of RNA molecules in the sample. Linear regressions for control mRNA input and NanoString positive controls are displayed in Figure 1C.

We found that the temporal variation in transcript abundance for individual genes was large, with some genes changing by over three orders of magnitude in under an hour (Figure 1D). In addition, the difference between the most and least abundant transcript within a single time point was four orders of magnitude. In NC10, there were 7.97×10^7 copies of *dhd* and 2.63×10^3 copies of *pnr*, a fold difference of $>30,000$ (Figure 1D). The change in expression for single genes and the differences in expression between various genes further reinforce our division of the MZT into 10 time points to capture rapid changes and highlight the dynamic nature of embryonic development during this time period.

To validate the accuracy of the NanoString instrument, we performed qPCR on two embryonic genes included in the study. We selected *snail* at peak expression during NC14C as a representative of expression level of many genes during this time, and *bicoid* during NC14D when the majority of transcripts have been degraded, to validate the ability of NanoString to detect rare transcripts (Figure 1E). We extracted total RNA from single embryos using the same method as NanoString experiments and the same exogenous mRNA spikes to quantify the number of transcripts. Using qPCR, we calculated 6566 ± 72 *bcd* transcripts present at NC14D, and 6458 ± 320 using NanoString, a difference of 1.68% (Figure 1F). For *sna*, we calculated $1,472,568 \pm 3681$ transcripts in the embryo during NC14C using qPCR, and $1,442,597 \pm 71,409$ transcripts using NanoString, a difference of 2.04% between qPCR and NanoString (Figure 1G). Because of the essentially identical values calculated with qPCR and NanoString, we concluded that our use of external mRNAs with NanoString to quantify all genes in the dataset is accurate.

Dynamic change between nuclear cycles is highly variable

When measuring the overall positive and negative change in transcript abundance from one nuclear cycle to the next, we noticed that the transition from NC14A to 14B is the most dynamic in the time course. Between NC14A and 14B, the greatest increase in transcription and greatest amount of degradation both occur, measured as positive or negative relative change for all genes from the previous nuclear cycle (Figure 2, A and B). The average fold increase for genes between NC14A and 14B was 5.6 ± 1.2 , while the average fold increase between all other NCs was 1.9 ± 0.2 . The decrease from NCs 14A to 14B was slightly less pronounced, at 3.0 ± 0.8 fold, compared to 1.6 ± 0.1 for all other NCs.

Of the genes with the greatest increase from NC14A to 14B, the majority are Dorsal targets expressed in the mesoderm or

neurogenic ectoderm, as well as genes also expressed in the dorsal ectoderm as part of the TGF- β pathway (Figure 2C). Most of the genes that rapidly decrease between NCs 14A and 14B are maternally deposited transcripts or are zygotically expressed and refined from broad to narrow patterns (Figure 2D). Purely maternal genes *dhd* and *yl* were among the most reduced transcripts, as well as zygotically refined genes *zen*, *scw*, and *hb*.

Surprisingly, the genes *bcd* and *spz*, both commonly thought of as purely maternal, showed evidence both of degradation of maternal products and zygotic transcription. Transcript counts for both *bcd* and *spz* first increased, then declined sharply between NCs 14A and 14B, indicating a quick burst of zygotic transcription as maternal products were being degraded (Figure 3A). The number of transcripts remains at a higher level than the minimum counted at the maternal-to-zygotic switch point for three or four additional time points, adding more weight to the finding that there is new embryonic transcription of these genes. *In situ* hybridizations using intronic probes show that there is in fact zygotic transcription of *bcd* detected as early NC11 (Figure S2), with dots of nascent nuclear signal visible in many nuclei throughout the embryo. Since maternal *bcd* is spliced and mature before the egg is laid, signal from intronic probes must indicate new zygotic transcription. It is possible that embryonic transcription is needed to maintain the correct level of protein if mRNA degradation occurs too quickly. This finding provides a new insight into the transcription and regulation of two genes and shows the strength of the NanoString system to acquire highly sensitive data that can be validated by other traditional experimental methods.

In addition to the change between nuclear cycles being highly variable, the switch from maternal to zygotic control is variable for genes that are both maternally deposited and zygotically transcribed. We define the maternal-to-zygotic switch point as the time when degradation of maternal input is overwhelmed by zygotic transcription and counts increase. We included 19 dual maternal and zygotic genes in the study and found that the maternal switch points occur as early as NC11 and as late as NC14A (Figures 3, A–C). Both dual switching AP genes included, *bcd* and *hb*, switch at NC12 (Figure 3A) along with seven other DV genes; however, DV genes *med*, *E(spl)m8*, and *sax* switch at NC11, *spi* and *cic* switch at early NC13, and *Neu3* and *pnt* switch at NC14A. The ubiquitous transcription factors *zld* and *Su(H)* have switch points at NCs 11 and 12, respectively. Because they are ubiquitous, we calculated the number of transcripts per nucleus or cell (for precellularized or postcellularized embryos, depending on nuclear cycle) in addition to the number of transcripts per embryo. Overall, maximum expression for *zld* and *Su(H)* occurs at NCs 14D and 14B, respectively, but when number of transcripts are divided by number of nuclei or cells present, transcripts are most abundant at NC10 (Figure 3D). This is consistent with studies showing that *zld* acts as an early activator of expression, with effects from lack of *zld* transcripts observed much earlier than NC14 (Nien

Figure 2 Dynamic change between nuclear cycles. (A and B) Average fold increase or decrease for genes changing between each nuclear cycle. The transition from nuclear cycle 14A to nuclear cycle 14B is the most dynamic in the entire time course, both in terms of the overall increase and decrease in number of transcripts detected for genes. Between these two time points, the amount of transcripts for some genes increases >50-fold in around 15 min. Error bars represent SEM. (C) There are 17 genes with a ≥ 5 -fold increase between nuclear cycle 14A and 14B, most of which are direct Dorsal targets in the mesoderm and ventral ectoderm, or targets of the TGF- β pathway. (D) There are seven genes with a ≥ 2 -fold decrease in this period, with genes maternally deposited and being degraded (blue), broadly expressed and being spatially refined (red), or both maternally deposited and zygotically transcribed before being degraded (purple).

et al. 2011). Robust transcription late in the time course is able to compensate for nuclear division and dilution of transcripts, and the number of transcripts per cell for both *zld* and *Su(H)* increase during NC14.

The relative rate of transcript degradation between each nuclear cycle follows the pattern of diversity observed in maternal-to-zygotic switch points, in that there is a wide range of rates at which maternal transcripts are degraded. We computed relative degradation between maternal genes by calculating the percentage of transcript decrease for each gene at nuclear cycle transitions and then comparing rates between genes. Degradation rates differ by up to 31.9% between genes, and degradation occurs until NC14A for some genes.

Zygotic genome activation and mesoderm gene network properties

The mesoderm presents an opportunity to study a set of genes that is spatiotemporally coactivated. We selected the genes *twi*, *sna*, *htl*, *hbr*, *NetA*, and *mes3*, which are all dependent on the binding of the transcription factor Dorsal for their expression. When the transcripts per embryo for the mesoderm genes are compared, it is clear that there is a specific rank order of abundance maintained throughout the time series (Figure 4A). *twi* is more than twice as abundant as the next gene, *mes3*, and more than seven times as abundant as the weakest gene, *htl*. All six mesoderm genes have similar boundaries on the DV axis (Figure 4, C'–F'), but have different boundaries on the AP axis (Figure 4, C–F). The *twi*

domain extends to the anterior and posterior poles of the embryo, while the *htl* domain is found in the middle $\sim 75\%$ of the AP axis. We counted the number of nuclei expressing all six mesoderm genes and determined the number of transcripts per nucleus. Even after normalizing for number of nuclei, the rank order of abundance remains the same for all six genes throughout the time series, although several genes that were differentially expressed in whole-embryo counts are more similarly expressed in individual nucleus counts. In late NC13, there are $\sim 25\%$ more *twi* than *sna* transcripts in the whole-embryo count, and the difference between the two genes drops to $<1\%$ in per-nucleus counts for a short time; however, the order is established again in NC14 (Figure 4, A and B). A similar change of 20% more *hbr* than *htl* in NC14 for the entire embryo drops to $<3\%$ per cell. Still, the rank order remains the same even when transcripts per cell are calculated. NCs 10 and 11 were difficult to estimate, since robust patterns do not appear until NC12; therefore, we did not include the earliest two time points in the per-nucleus calculations.

It is also clear that transcription of mesoderm genes is biphasic. In NCs 10–13, there is a moderate and steady increase for each of the six genes. In NC14, the increase in number of transcripts becomes much more rapid. Since all six mesoderm genes depend on Dorsal and Twist for activation, and Dorsal is maternally deposited, we analyzed embryos from *twi*[−] flies in an attempt to explain the rapid increase in transcription observed in NC14. We selected late

Figure 3 Diversity in maternal-to-zygotic switch points. (A) AP axis genes *hb* and *bcd* and (B) DV axis genes *shn*, *Neu3*, and *pnt* are both maternally deposited and zygotically transcribed. (C) The broadly acting transcription factors *zld* and *Su(H)* are both maternally deposited and zygotically expressed, with the zygotic activation occurring early at NC11 and 12 for *zld* and *Su(H)*, respectively. (D) Despite the overall increase in number of transcripts for both *zld* and *Su(H)*, the highest number of copies per nucleus occurs at NC10, before the maternal transcripts are completely degraded and zygotic transcription takes place. Transcription of both genes is strong enough, however, to cause a slight increase in number of transcripts per cell during NC14. (E) A timeline of maternal-to-zygotic switch points, with the number of each class of gene that switches at every time point. All error bars represent confidence interval $P \leq 0.001$.

NC13, 14C, and gastrulation for the *twi*⁻ analysis, which cover early, peak, and declining Twist activation. We found that in late NC13 *twi*⁻ embryos, the average expression of mesoderm genes was $76.4\% \pm 11.6\%$ of wild type, indicating that Dorsal activation accounts for $\sim 76\%$ of transcription at that time point, with some variability between genes, while Twist supports the rest of the activating input. At NC14C, the average expression level of mesoderm genes in *twi*⁻ embryos was $22.5\% \pm 8.5\%$ that of wild type. This drastic drop suggests that Twist is responsible for $>77\%$ of the expression of mesoderm genes at this time. During gastrulation, the average expression of mesoderm genes slightly recovered to $55.9\% \pm 3.7\%$ of wild-type levels, implying that Twist is responsible for less than half of the activation. When the data from *twi*⁻ embryos is plotted with wild-type data, it is evident that without Twist activation, the transcription rate of the mesoderm genes matches the early transcription rate, when Dorsal is the predominant activating transcription factor (Figure 4, G–J). *In situ* hybridizations show the reduction in expression for mesoderm genes in *twi*⁻ embryos (Figure 4 K–N). Therefore, the input of Twist is responsible for the rapid increase in transcription observed for the mesoderm genes during nuclear cycle 14.

Sequential activation of the TGF- β signaling pathway and compensatory transcription

The TGF- β signaling pathway is one of the best-studied signaling pathways in *Drosophila*, and model organisms in general, and because the components are well known, presents an opportunity to observe how the MZT activates a complete signaling pathway (Wu and Hill 2009; Akhurst and Padgett 2015). We included 18 members of the TGF- β pathway, as well as others peripherally related. The two primary ligands are Dpp and Scw: both purely zygotically transcribed. While peak TGF- β signaling takes place in the dorsal ectoderm, both *scw* and *dpp* are initially expressed in broader regions of the embryo. The expression of *dpp* extends to the ventral midline during NC13 and the expression of *scw* is ubiquitous starting as early as NC11, and both genes refine to the dorsal ectoderm during NC14. Our NanoString data confirms this initial broad expression and subsequent refinement of both *dpp* and *scw* (Figure 5A). Furthermore, both *scw* and *dpp* decrease at very similar rates from NC14B onwards, including a pause in decreasing from NC14C to 14D, when they are both in the last stage of refining to their final expression domain.

We included six TGF- β targets in the study and found that they are all strongly activated beginning in NC14. We

Figure 4 Mesoderm gene expression and transcription rates. (A) Expression profiles of the mesoderm genes *twi*, *htl*, *mes3*, *sna*, *NetA*, and *hbr*. (B) Number of transcripts per cell was calculated by dividing the absolute number of transcripts by number of cells expressing each gene. (C–F) *In situ* hybridization using riboprobes against mesoderm genes *twi*, *sna*, *htl*, and *NetA*, showing their respective expression domains laterally and dorsally. (G–J) Expression of *twi*, *sna*, *htl*, and *NetA* in *twi*[−] embryos, with mutant expression data collected at late NC13, NC14C, and gastrulation. Dashed linear regression trend lines show trajectory of expression without *twi*. (K–N) *In situ* hybridizations using riboprobes to *twi*, *sna*, *htl*, and *NetA* in *twi*[−] embryos. All error bars represent confidence interval $P \leq 0.001$. Embryos are staged to NC14C.

separated TGF- β targets into two classes, based on how they are activated in NC14. Genes *pnr*, *hnt*, and *Doc1* are expressed in a gradually increasing manner throughout NC14, until gastrulation when the rate of transcription levels off (Figure 5B). In contrast, *Race*, *tup*, and *ush* increase very quickly at the beginning of NC14 and reach a plateau as early as NC14B or 14C (Figure 5B).

The TGF- β targets are expressed in the same general domain of the embryo, but the exact patterns differ between the genes. We counted the number of cells expressing the genes *ush*, *Race*, and *hnt* for NCs 14C, 14D, and at the onset of gastrulation. We focused on these three genes because they are expressed purely along the DV axis, unlike the other three that are expressed in AP-modulated patterns as well, and these time points because they fall during the peak of TGF- β signaling, when the genes are expressed in their final

domains. TGF- β target expression during earlier time points is still developing and final patterns are not yet established. When the whole-embryo transcript levels for the three genes are compared, *ush* is always the most abundant, with *Race* at ~60% of the *ush* levels and *hnt* at ~22% of *ush* levels (Figure 5F). However when the number of transcripts per each cell is calculated based on expression domain (Figure 5, C–E), the results change drastically. The difference between *Race* and *ush* drops to 1–4% depending on the time point, and the difference between *hnt* and *Race* and *ush* drops to 6–15% depending on time point (Figure 5G). The similarity in number of transcripts expressed in each cell for *Race*, *ush*, and *hnt* suggests that the genes respond in a comparable way to common transcriptional activators. There may be repressors that define the extent of each gene's patterns, but in the cells where each of the genes is active, the genes are transcribed at similar levels.

Figure 5 Activation and properties of the TGF- β signaling pathway and targets. (A and B) Expression profiles for TGF- β ligands *scw* and *dpp* (A) and TGF- β target genes *ush*, *tup*, *Race*, *pnr*, *hnt*, and *Doc1* (B) showing number of transcripts per embryo. (C–E) *In situ* hybridizations using riboprobes showing expression patterns for *ush*, *Race*, and *hnt*, both laterally and dorsally. (F) Total number of transcripts per embryo during peak expression for *ush*, *Race*, and *hnt*. (G) Number of transcripts per cell for *ush*, *Race*, and *hnt*. All error bars represent confidence interval $P \leq 0.001$.

Discussion

Our use of NanoString technology combined with our fine time scale developmental window has provided a novel way to examine transcription during the MZT in *Drosophila*. The dynamic change between NCs reveals new insights into the development of *Drosophila* embryos. The transition from NC14A to 14B is the most dynamic in the study, and is unique for three reasons. First, the concentration of Dorsal in cells is at its highest level at this time point, allowing activation of genes on the dorsal edges of Dorsal gradient that were not activated by lower levels in previous NCs. Second, this is the first time that transcription proceeds uninterrupted for >15 min, allowing a greater ramp-up time for highly expressed genes to accumulate to levels not reached before. Lastly, the combination of increased Dorsal concentrations and more time available for transcription allows novel gene interactions and cell signaling to take place within the DV gene network that were not possible before, further increasing the number of genes expressed and the levels they are expressed.

One exception to the biphasic transcription modes for mesoderm genes is *twi*, which begins its increase in expression rate at the end of NC13, slightly earlier than the other genes. The combination of this earlier increase in transcription rate for *twi*, the overall highest abundance of *twi*, and the

role of Twist as a master coactivator with Dorsal in the mesoderm lead us to hypothesize that the biphasic transcription for mesoderm genes is due to the input of *twi* in the gene network. In *twi*[−] embryos, when there would usually be the lowest endogenous abundance of *twi*, other genes change the least, and where there would usually be the highest endogenous level of *twi*, other genes are affected to the greatest degree observed. We therefore conclude that the moderate expression observed from NCs 10–13 is due to the input of Dorsal, while the exponential increase in expression in NC14 is due to the input of Twist and a combinatorial effect of the Dorsal–Twist feed-forward loop. With *twi* as a top-level activator in the mesoderm and early target of Dorsal, high levels of *twi* are needed so Twist can robustly bind its targets in every cell where it is needed (Sandmann *et al.* 2007). It is inline with this prevailing view, therefore, that *twi* is consistently the most abundant mesoderm gene quantified.

Two studies have quantified the number of transcripts for two genes included in this study, using FISH to estimate the number of transcripts (Boettiger and Levine 2013; Petkova *et al.* 2014). One study of *bcd* transcripts prior to the syncytial blastoderm stage and NC10 found 890,000 transcripts. Our study found 824,064 transcripts during NC10, at the closest stage to the embryos used in the previous study; however, significant transcript degradation occurs between the time point in the previous study and NC10. A second previous

study quantified *sna* transcripts and found a maximum of ~250 transcripts per nucleus during NC13 and 200 transcripts per cell during NC14, while our data show a maximum of ~550 transcripts per nucleus in NC13 and ~1000 transcripts per cell during peak expression at NC14C, a two- to fivefold difference.

Using FISH to count single points of fluorescence can be challenging, with probe design and microscopy techniques affecting the counts (Femino *et al.* 1998; Raj *et al.* 2008; Lubeck and Cai 2012). In addition, the combination of dense points of fluorescence signal making it difficult to distinguish individual spots, and the use of a threshold to exclude fluorescent signal, may reduce the number of transcripts counted and account for the differences between our quantification and the numbers calculated for *bcd* and *sna*. The authors of the *sna* study counted only cytoplasmic signal, excluding nuclear transcripts, which might have reduced the count and, by design, did not account for active transcription. One factor that could slightly inflate the number of *sna* transcripts per cell we calculated is a low level of background transcription in nonmesoderm cells. If *sna* is expressed at a very low level in cells outside the mesoderm, our calculations would attribute these transcripts to mesoderm cells and slightly increase our quantification. This would lead to a negligible increase, since the transcriptional activity of cells expressing *sna* is so much stronger than nonmesoderm cells that *sna* transcripts are undetectable using standard *in situ* hybridization.

Furthermore, our qPCR data reinforce the accuracy of our quantification method and postcollection data analysis and processing. Previous foundational studies have compared changes in gene expression using NanoString and qPCR for different time points in the development of sea urchin embryos, and it was found that the relative fold changes calculated between time points were highly correlated between NanoString and qPCR data (Geiss *et al.* 2008; Materna *et al.* 2010).

The diversity observed for both the maternal-to-zygotic switch point and degradation rate can be explained by the increasing concentration of Dorsal in nuclei during successive NCs. As the concentration of Dorsal increases, the activation of target genes occurs at different rates and times, depending on whether genes depend directly on Dorsal, the concentration of Dorsal required, or the necessity of an intermediate gene. It is possible that degradation rates alone for genes are much more similar than we have observed, but since genes are activated at different rates and times, the varying influx of embryonic transcripts may cause the observed degradation rate to differ from the basal level.

Although NanoString technology does not provide spatial information on gene expression *a priori*, interesting trends or new insights from this data can be validated using other methods. In the case of mesoderm genes, using NanoString we determined that a rank order of abundance is established early in development and is maintained robustly through the time series. The rank order of genes was first observed for the entire embryo, meaning that spatial variations were not

originally taken into account, but remained the same after transcripts were normalized for number of cells, indicating that the order is established and maintained at the level of gene regulation (e.g., enhancer and gene network properties). This combination of NanoString data and spatial information strengthens the finding and provides an example of how NanoString can be used to investigate multiple genes simultaneously and integrate with other methods.

Of the six TGF- β targets studied, *Race*, *ush*, and *hnt* are expressed only in the dorsal ectoderm, while *pnr*, *tup*, and *Doc1* are also regulated along the AP axis, expressed in stripes or laterally toward the midline of the embryo. The TGF- β targets respond to activation in two distinct ways, with half of the genes rapidly transcribed between NCs 14A and 14B and quickly reaching a steady state, and half of the genes being continuously transcribed at moderate rates until gastrulation. The different modes of transcriptional activation do not appear to correlate with the genes based on expression patterns, indicating that there could be an unknown factor involved in rapidly activating one set of genes, just as we have shown that *twi* rapidly activates mesoderm genes. Once the TGF- β targets are activated and reach their peak expression, the maintenance of final levels might no longer depend on this initial activating signal, just as the mesoderm genes depend on *twi* the least at gastrulation, after peak expression. While these TGF- β target genes are diverse in terms of function, the convergence of transcript abundance in each cell, we propose, may demonstrate a unique property of the signaling pathway to integrate changing levels of input to maintain stable and reliable transcription of target genes. This property can be contrasted with the six mesoderm genes, where even after normalizing for number of nuclei expressing each gene, many differences in expression remained present throughout the time course. This difference may exist because the six mesoderm genes are at the top level of signaling pathways (e.g., *htl* FGF receptor) while the TGF- β targets are at the output level. Varying levels of top-level input signal may be integrated (i.e., coordinated) to provide a similar output level of many downstream target genes within a tissue.

We have demonstrated the use of NanoString as a new technology to precisely quantify transcripts and create a fine-scale time course of *Drosophila* embryonic development. In addition to being the first large-scale quantification during *Drosophila* development, this study has provided new insights into the sequential activation of gene regulatory networks and suggested that network properties regulate levels of transcription for clades of genes. We believe the most promising future use of NanoString is in the characterization of mutant phenotypes and accurately measuring changes in expression of large numbers of genes in mutant backgrounds, as we show with *twi* mutant data.

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Supporting Information

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Quantitative Single-Embryo Profile of *Drosophila* Genome Activation and the Dorsal–Ventral Patterning Network

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Figure S1. Background hybridization rate for all NanoString probes. Raw background counts are an average of three RNA-negative runs and sorted from lowest to highest counts.

Figure S2. In situ hybridization with probes for *bcd* introns, showing an embryo in NC11

Table S1. A list of genes included in probe set including expression timing, protein function, and expression domain

Gene	Symbol	FlyBase ID	Timing	Class	Expression Domain
<i>Ankyrin-repeat, SH3-domain, and Proline-rich-region containing Protein</i>	<i>ASPP</i>	FBgn0034606	Zygotic	Protein binding/modifying	Mesoderm
<i>CIAPIN1 ortholog</i>	<i>CIAPIN1</i>	FBgn0001977	Ubiquitous	Iron-Sulfur Cluster Binding	Ubiquitous
<i>Delta</i>	<i>Dl</i>	FBgn0000463	Maternal + Zygotic	Transcription Factor	Ubiquitous (--> Ventral)?
<i>Dorsocross1</i>	<i>Doc1</i>	FBgn0028789	Zygotic	Transcription Factor	Dorsal Ectoderm/Amnioserosa
<i>Epidermal growth factor receptor</i>	<i>Egfr</i>	FBgn0003731	Zygotic	Cell Membrane Receptor	Ubiquitous with A-P modulation
<i>β-galactosidase</i>	<i>LacZ</i>	N/A	Control	Negative Control	None
<i>Drop</i>	<i>Msh/Dr</i>	FBgn0000492	Zygotic	Transcription Factor	Dorsal Ectoderm with A-P Modulation
<i>Notch</i>	<i>N</i>	FBgn0004647	Maternal + Zygotic	Cell Membrane Receptor	Ubiquitous
<i>Netrin-A</i>	<i>NetA</i>	FBgn0015773	Zygotic	Secreted: Laminin	Mesoderm
<i>Meltrin</i>	<i>Neu3/Meltrin</i>	FBgn0265140	Maternal + Zygotic	Metalloprotease	Neurogenic Ectoderm
<i>Angiotensin converting enzyme</i>	<i>Race/Acne</i>	FBgn0012037	Zygotic	Metallopeptidase	Dorsal Ectoderm/Amnioserosa
<i>Ribosomal protein L32</i>	<i>RpL32</i>	FBgn0002626	Ubiquitous	Ribosomal Protein	Ubiquitous
<i>Ribosomal protein L7-like</i>	<i>RpL7-like</i>	FBgn0032404	Ubiquitous	Ribosomal Protein	Ubiquitous
<i>SoxNeuro</i>	<i>SoxN</i>	FBgn0029123	Zygotic	Transcription Factor	Neurogenic Ectoderm
<i>Suppressor of Hairless</i>	<i>Su(H)</i>	FBgn0004837	Maternal + Zygotic	Transcription Factor	Maternal --> Ubiquitous
<i>Toll</i>	<i>Tl</i>	FBgn0262473	Maternal + Zygotic	Cell Membrane Receptor	Maternal --> Ubiquitous
<i>wnt inhibitor of Dorsal</i>	<i>wntD</i>	FBgn0038134	Zygotic	Secreted: Ligand	Faint Mesoderm, Strong Ventral Midline
<i>Yellow fluorescent protein</i>	<i>YFP</i>	N/A	Control	Negative Control	None
<i>argos</i>	<i>aos</i>	FBgn0004569	Zygotic	Secreted: Ligand	Dorsal Ectoderm with A-P Modulation
<i>bicoid</i>	<i>bcd</i>	FBgn0000166	Maternal + Zygotic	Transcription Factor	Maternal --> Anterior
<i>brinker</i>	<i>brk</i>	FBgn0024250	Zygotic	Transcription Factor	Ventral Ectoderm
<i>capicua</i>	<i>cic</i>	FBgn0262582	Maternal + Zygotic	Transcription Factor	Maternal --> Dorsal Ectoderm
<i>deadhead</i>	<i>dhd</i>	FBgn0011761	Maternal	Thioredoxin	Maternal --> Pole Cells
<i>decapentaplegic</i>	<i>dpp</i>	FBgn0000490	Zygotic	Secreted: Ligand	Dorsal Ectoderm/Amnioserosa
<i>even skipped</i>	<i>eve</i>	FBgn0000606	Zygotic	Thioredoxin	A-P Pair Rule
<i>flagrante delicto Y</i>	<i>fdy</i>	FBgn0265047	Zygotic	RNA Binding	Unknown
<i>grainy head</i>	<i>grh</i>	FBgn0259211	Zygotic	Transcription Factor	Ventral Ectoderm
<i>hunchback</i>	<i>hb</i>	FBgn0001180	Maternal + Zygotic	Transcription Factor	Maternal --> Anterior
<i>pebbled</i>	<i>hnt/peb</i>	FBgn0003053	Zygotic	Transcription Factor	Dorsal Ectoderm/Amnioserosa
<i>heartless</i>	<i>htl</i>	FBgn0010389	Zygotic	Cell Membrane Receptor	Mesoderm
<i>inflated</i>	<i>if</i>	FBgn0001250	Zygotic	αPS2 Integrin	Mesoderm
<i>intermediate neuroblasts defective</i>	<i>ind</i>	FBgn0025776	Zygotic	Transcription Factor	Ventral Ectoderm
<i>Enhancer of split m8, helix-loop-helix</i>	<i>m8/E(spl)m8- 1LHF</i>	FBgn0000591	Maternal + Zygotic	Transcription Factor	Maternal --> Ventral Midline
<i>Mothers against dpp</i>	<i>mad</i>	FBgn0011648	Maternal + Zygotic	Signal Transduction/Transcription Factor	Maternal --> Ubiquitous
<i>Medea</i>	<i>med</i>	FBgn0011655	Maternal + Zygotic	Signal Transduction/Transcription Factor	Maternal --> Ubiquitous
<i>Insulin-like peptide 4</i>	<i>mes3/Ilp4</i>	FBgn0044049	Zygotic	Secreted: Ligand	Mesoderm
<i>ocelliless</i>	<i>otd/oc</i>	FBgn0004102	Zygotic	Transcription Factor	Gap Gene Anterior
<i>phantom</i>	<i>phm</i>	FBgn0004959	Zygotic	Cytochrome P450	Ubiquitous --> Mesoderm
<i>pannier</i>	<i>pnr</i>	FBgn0003117	Zygotic	Transcription Factor	Dorsal Ectoderm/Amnioserosa
<i>pointed</i>	<i>pnt</i>	FBgn0003118	Maternal + Zygotic	Transcription Factor	Maternal --> Ventral Ectoderm, Anterior & Posterior
<i>punt</i>	<i>put</i>	FBgn0003169	Maternal + Zygotic	Cell Membrane Receptor	Maternal --> Ubiquitous
<i>pyramus</i>	<i>pyr</i>	FBgn0033649	Zygotic	Secreted: Ligand	Neurogenic Ectoderm
<i>rhomboid</i>	<i>rho</i>	FBgn0004635	Zygotic	Intramembrane Serine Protease	Ventral Ectoderm
<i>saxophone</i>	<i>sax</i>	FBgn0003317	Maternal + Zygotic	Cell Membrane Receptor	Maternal --> Ubiquitous
<i>screw</i>	<i>scw</i>	FBgn0005590	Zygotic	Secreted: Ligand	Ubiquitous --> Dorsal Ectoderm/Amnioserosa
<i>schnurri</i>	<i>shn</i>	FBgn0003396	Maternal + Zygotic	Transcription Factor	Maternal --> Dorsal Ectoderm
<i>single-minded</i>	<i>sim</i>	FBgn0004666	Zygotic	Transcription Factor	Ventral Midline
<i>slit</i>	<i>sli</i>	FBgn0264089	Zygotic	Secreted: Ligand	Neurogenic Ectoderm
<i>snail</i>	<i>sna</i>	FBgn0003448	Zygotic	Transcription Factor	Mesoderm
<i>short gastrulation</i>	<i>sog</i>	FBgn0003463	Zygotic	Secreted: Protein Binding	Neurogenic Ectoderm
<i>spitz</i>	<i>spi</i>	FBgn0005672	Maternal + Zygotic	Secreted: Ligand	Maternal --> Ubiquitous
<i>spatzle</i>	<i>spz</i>	FBgn0003495	Maternal	Secreted: Ligand	Maternal
<i>Star</i>	<i>star/S</i>	FBgn0003310	Maternal + Zygotic	Transmembrane Protein	Maternal --> Ventral Midline
<i>stumps</i>	<i>stumps</i>	FBgn0020299	Zygotic	Signal Transduction	Mesoderm
<i>thisbe</i>	<i>ths</i>	FBgn0033652	Zygotic	Secreted: Ligand	Neurogenic Ectoderm
<i>tinman</i>	<i>tin</i>	FBgn0004110	Zygotic	Transcription Factor	Mesoderm with A-P Modulation
<i>thickveins</i>	<i>tkv</i>	FBgn0003716	Maternal + Zygotic	Cell Membrane Receptor	Maternal --> Mesoderm & Dorsal Ectoderm with A-P Modulation
<i>tolloid</i>	<i>tld</i>	FBgn0003719	Zygotic	Metallopeptidase	Dorsal Ectoderm
<i>twisted gastrulation</i>	<i>tsg</i>	FBgn0003865	Zygotic	Protein binding/modifying	Dorsal Ectoderm with A-P Modulation
<i>tailup</i>	<i>tup</i>	FBgn0003896	Zygotic	Transcription Factor	Dorsal Ectoderm/Amnioserosa
<i>twist</i>	<i>twi</i>	FBgn0003900	Zygotic	Transcription Factor	Mesoderm
<i>u-shaped</i>	<i>ush</i>	FBgn0003963	Zygotic	Transcription Factor	Dorsal Ectoderm/Amnioserosa
<i>vein</i>	<i>vn</i>	FBgn0003984	Zygotic	Secreted: Ligand	Neurogenic Ectoderm
<i>ventral nervous system defective</i>	<i>vnd</i>	FBgn0261930	Zygotic	Transcription Factor	Ventral Ectoderm
<i>yolkless</i>	<i>yl</i>	FBgn0004649	Maternal	Cell Membrane Receptor	Maternal
<i>zerknüllt</i>	<i>zen</i>	FBgn0004053	Zygotic	Transcription Factor	Dorsal Ectoderm
<i>zelda/vielfaltig</i>	<i>zld/vfl</i>	FBgn0259789	Maternal + Zygotic	Transcription Factor	Maternal --> Ubiquitous
<i>Rm62</i>	<i>Rm62</i>	FBgn0003261	Ubiquitous	RNA Binding	Ubiquitous
<i>B. subtilis dap</i>	<i>dap</i>	N/A	Control	Positive Control	None
<i>B. subtilis lys</i>	<i>lys</i>	N/A	Control	Positive Control	None
<i>B. subtilis phe</i>	<i>phe</i>	N/A	Control	Positive Control	None
<i>B. subtilis thr</i>	<i>thr</i>	N/A	Control	Positive Control	None

Table S2. NanoString probe sequences. Genes *NetA*, *sog*, and *vn* have two probes each.

Symbol	Probe sequence
<i>ASPP</i>	GTCGGCATCGACTGCTAATAAGACGCAAACGGCCACCTTTGGACTGCAATCCCTTAACATCAACGACAATTTACCCATCAAAGCGAAGCCACTGACCATC
<i>CIAPIN1</i>	CTGCATTTGGTGTCCTACATCGGTCCGGCGGGCTAGTTTGCTGCAGGAGATCAAGCTATCCGGATTCCAAACTGTGCGGAGGACTCTCCAGATGCTTTGA
<i>DI</i>	CTGGCAGCAACAGCGGTCTCACCTTCGATGGCGGCAACCCGAATATCATCAAAAAACCTGGGACAAGTCGGTCAACAACATTTGTGCCTCAGCAGCAGC
<i>Doc1</i>	CAGCGCCAGGATTTTGAGCAGGACTCAACGGGTAGTGTTTCAGCTTATCCGTGGAGGAGGAGAAGTATGCCTTGTTTCGTCACTTATTCAAAAATGTACACGCTGGAGA
<i>Egfr</i>	AAACTACAAATCATTTCGCGGACGCACGCTGTTTCAGCTTATCCGTGGAGGAGGAGAAGTATGCCTTGTTTCGTCACTTATTCAAAAATGTACACGCTGGAGA
<i>LacZ</i>	CATCAAAAAATGGCTTTTCGCTACCTGGAGAGACGCGCCCGCTGATCCTTTGCGAATACGCCACGCGATGGGTAACAGTCTTGGCGGTTTCGCTAAATAC
<i>Msh/Dr</i>	CCCTTCGGACCGCCTGGCATGTTTCCGGGAGCAGGATTCGGTGGAGATGCCAACGAACCGCCGCGGATCAAGTGCAACCTGCGGAAGCACAAAGCCCAACC
<i>N</i>	GATGGAAC TTGCCACGACAAGATCAATGGCTTTAAATGCAGTTGTGCCCTGGGCTTTACGGGCGCACGATGTCAAATCAATATAGACGACTGTCAGTCGC
<i>NetA (probe 1)</i>	TCCTGTACAGCCAGTTTAAAGCATTTGTGAACAATTTACGTACTAGGGCCGTCTCGAAATCAAAACAGAGCGCCAGTAAGCAATAATTTATGTGTAAAGAAC
<i>NetA (probe 2)</i>	CGCGCCTGCATTCCGGACTTTGTGAACGCCGCCTACGATGCTCCAGTGGTGGCCAGTTCCACGTGCGGATCGTCGGGTGCGCAGCGATATTGCGAGTACC
<i>Neu3/Meltrin</i>	ACGAGATGGGGCACAACTTTGGCATGGAGCACGATACATCGGATTGTCA TTGTGCGGGATGAGAAGTGTGTGATGGCTGCCTCGAGTACCTCGTTTTATTCC
<i>Race/Acne</i>	TCTACGACAAGGCCGGAACCGCCGTGCGATCTCAATTGCAACGCTACGTGGAGCTCAACACCAAGGCAGCCAAACTGAATAACTTTACTTCTGGTGCCGA
<i>RpL32</i>	TGCCCACCGGATTCAAGAAGTTCCTGGTGCACAACGTGCGCGAGCTGGAGGTCTGTCTATGCAGAACC
<i>RpL7-like</i>	GTGTCATCTGTCTGGAGGATATAATCCACGAAATTTGCACAGTGGGTCCAACTTCGCCGCTGTAAATGAGTTTCTGTGCGCCTTCACGTTGTCCAGTCC
<i>SoxN</i>	TGGGCCAGTCGCACCTGACCCACTCGCAGCTGAGCCATCACAACCACCATCATCACCACATGAGCGCCCATATCGCGGCCAGCCAGAGTCCCAATCCGCT
<i>Su(H)</i>	GAAGCCGACGATCCCGTTTCTCAGCTGCACAAATGCGCCTTCTACATGAAGGATACGGATCGGATGTATCTCTGTTTGTGCGAGGAGAAAATCATA
<i>Tl</i>	GGGCAACCTTGTGACCCTCGTCATGAGCAGGAATCGACTGCGTACCATCGATTCCAGAGCCTTTGTAAGCACAAATGGACTGCGACATTTGCATTTGGAT
<i>wntD</i>	GCGGTACTAAAGCCATTGCAAGCCATTGCCAGGATCTCCTCCAAATGTACGACGATGCCATCCAACTAGAAGGAGCCAGTAGCAATCTAAAGATTATGT
<i>YFP</i>	GAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGCCACAAGCTGGAGTACA
<i>aos</i>	ATGTCGTTGTCCTGAATCGAATCGCATGCCCCAACAATGTGATCATCCATCATCACAGTCATTCTCTCGGGATCGGTGGATCCCTGAAGTACAGGAACTAC
<i>bcd</i>	TGAAGGGTCTGGACAAGAGCTGCGACGATGGCAGTAGCGACGACATGAGCACCGGAATAAGAGCCTTAGCAGGAACCGGAAATCGTGGAGCGGCATTTGC
<i>brk</i>	CCGCCGTCAAATGCAGTTGCACGAAAAGGATCCATCTGGTGTGGATCTCACTTTCGCAAACGTAAGGTCATCACTAGTCCGATGCAGCCGGATAAGATT
<i>cic</i>	GGCAGCTGCGGTGGCCAGGGCATATCATCCGATTTGCAGGGCGATATTATACCGCTGACAATTGACA
<i>dhd</i>	ATACTCCAGCAAGGCGGTGGTGCTCAAGATCGATGTGGACAAATTCGAGGAGCTGACGGAGCGCTACA
<i>dpp</i>	CAGCGAGCCCCGCCTCGTTCA
<i>eve</i>	AGACGCCGCCGCTGGATCTGCAGTCGTCGTCATCGCCGCACTCCTCCACGCTGTGCTCTCGCCAGTGGGATCCGATCACGCCAAGGTGTTTCGACCCGAG
<i>fdy</i>	CAACAACAACCAACGCTACGGAAACAAGGAGTCGATCGGAGAATTTCGAAACGAGTTGCCG
<i>grh</i>	AGATCAACATTGCGGTTCA
<i>hb</i>	CACCGCAAGTCGCACAGTTCTGTGTATCAGTACC
<i>hnt/peb</i>	CCATTACGGCGCCCAGTACTAATCCCAGCAGTTTGAAAACGATGATCGCTCAAGCCGAATATGTGGGAAAATCCCTCAAAGAAGTGGCCAGTTCACCGTT
<i>htl</i>	TAATCAATCGAGCACATCCAGTGCGGATTTGGATGATGGCGCCG
<i>if</i>	GTGCCAGCGTTCATATCGAGAAGGCTCGCGGTGAAGGTTTCGTTTCGAGGAGTCTTGGTCAGCAATAGCACCGATGCGGGTGACAAATTAAGTCCCAAACA
<i>ind</i>	CCATCCATACGCCCAGCTGTTTCGCCAGCAAGAGGAAGTCCAGTGGATT
<i>m8/E(spl)m8-HLH</i>	CTCCACACCTGGCATGAGCGTCGACCTGGGCAAATCGGTGATGACTCACCTGGGACGCGTCTACAAGA
<i>mad</i>	GAACAGTGGATTCAACTCGCACAGCTTGAGCACACGCAACACATCGGTGGG
<i>med</i>	CATCGCATACTTCAGAGTTGGACACGCAAGTGGGCGAGACCTTCAAGGTGCCGTCAGCGAAACCGA
<i>mes3/Ilp4</i>	CGCCGAAAGATGTGCGGCGAGGCTCTGATCCAGGCACTGGATGTGATTTGTGTTAATGGATTAC
<i>otd/oc</i>	TTTAGCAATCAGAATCAGGTCAACTACAACATGGGCCATTTCGGGCTACACGGCCTCCAATTTTGGTCTGTG
<i>phm</i>	CCTACCAGGCGCCGCCACAGTTTATCCCATTCTCGTCCGGCTATCGAATGTGTCCCGGCGAAGAGATGGCTCGCATGATACTCACGCTCTTTACGGGT
<i>pnr</i>	AACAATGCTTTCCCTTTACGGACAGACGACGACGCAACAGCAGCACCAGCAACATGGCCACAGCATGACATCATCCTCTGGACAGGCACATCTGAGTGC
<i>pnt</i>	GACAAGACGTGT
<i>put</i>	CGCCATCCGAACATCCTCGAATTCCTGGGCGTTGAGAAGCACATGGACAAGCCGGAATATTGGCTGATATCCACCTACCAGCATAACGGATCACTATGCG
<i>rho</i>	AAAGCCAAGTTGAGACAACAGCGAACCCCGAAGGTGAGACAGAGACAGTTACTACCGAAACTGTAATGCAGAATCGCAATCATATTGATGCTAATAATAT
<i>sax</i>	ATCCTAGTGATCTCCATCATTGAGATTGCCATCTTCGCCCTACGACCGCTACACAATGCCCGCCAGAAATTCGGGGCTACCCGTTCCGATTCCGTCGGATT
<i>scw</i>	GTTCTGGGTCCTTTTCTGGTCATCGCTCTGCTGGGCGCCGTGACCATCTTCTTCATTTCGTCGTAGCCATCGCAAGCGTCTGGCTGCCTCGCGAACCAAAC
<i>shn</i>	CAATAATCACATCGAGATGCCATATAACCAGAAGCGTCCTTTGAGCGAGCAAATGGAGATGATTGATATCCTGGATTTGGGTGACCGTCCGAGACGACAG
<i>sim</i>	CAGTCTGCAAAACAGCATTGGCTCTTCGGGTTTCGCAACCTAAAAGATTAGTGTGCTCCTTTACATCGCCGAAGCCGCTTCGACTACCAAAAGCAGGAA
<i>sli</i>	CGTGCAATTTGGGCCTCAGTCAGGTTGAGCTGACGGGCAACTCGATATTCGAGTACATACAACTACGATCAGGACGAGATGAATGCCATTTTGTGCTG
<i>sna</i>	ATAGTCTTCAGTAGCGCGGAGCAGAATGGAATTCTCATGTACGACGGCCAGGATGCTCATCTTGCAGTGGA
<i>sog (probe 1)</i>	TTGCCGCAATCATGCCAAAAACTACCGCTTCAAGTGCGACGAGTGCCAGAAGATGTACTCCACCTCGATGGGCCTGTCCAAGCACCGTCA
<i>sog (probe 2)</i>	TTGCTCATCATCGCCGACTGCTGATCGTCTGCTTGGCGGGCGTGACGGAGGGCCGCCGGCATGCGCCGCTCATGTTTCGAGGAGTCCGACACGGGCAGGCG
<i>spi</i>	GCAGTGCCCAGGATTCTCTGT
<i>spz</i>	AAACCTTCGATGCCTGGTACTGTTTGAACGATGCCATTTGCTTTGCGGTGAAGATAGCCGATCTACCGGTTTACAGCTGCGAGTGCGCGATTGGCTTTAT
<i>star/S</i>	TCAGACGAGCGATTCTTTTG
<i>stumps</i>	GCTGCCAGAAGTCCACGGGCATGGAGTCCCGGGCGAGAACATCTCCGCAACAGATCCAGCATCCGCACAGACAGCACCACCAGCAGCAGCACCATCACCA
<i>ths</i>	TACTGGCCATTTTGTGTGGATGT
<i>tin</i>	CAACCTGAACAACACACTAACGGAGGCTTCTCCCACGGCAACATCAACATCCAGCATCACGTCCGCAACAACCATGAAGAGACCCATACGGAAAGTTCACC
<i>tkv</i>	GGAGCATCTACGGTTCAGATGAGGGCGCCAAAAAAAAGGATAACAGCCAGGTGACCTCCTCGCGTTCCGAGCTGCGAAAAAACAGCATCAGTGGGAACAG
<i>tld</i>	CAACCTGAACAACACACTAACGGAGGCTTCTCCCACGGCAACATCAACATCCAGCATCACGTCCGCAACAACCATGAAGAGACCCATACGGAAAGTTCACC
<i>tsg</i>	GAACTGGATGTCATTACATTGCACAGAATCCACGTGTCGGCACTCGACGCTACATGGCTCCAGAAGTATTGAGTCAGCAGCTGGATCCCAAGCAGTTTG
<i>tup</i>	CCATGAAAAATGAAAGCGAAATTAGTTGTTCTCTGTGGGAGCTCTCTGGATGATGATGTTTTTCTGGTGGATTACGCCGAGGGCAGGCGACTGAGTCA
<i>twi</i>	AGCCGTCGTATCGCGAACATCGACCGTCTTAGCTCGATAGCCGTCCGATTTGCCTTTTTCGGATGCGAGAGACTCTTAAATCCCTACCATAGATCCAAAT
<i>ush</i>	GATGCACTTCCAAAACGCCTACAGACAAAGTTTCGAGGGCTACGAGCCGGCCAACTCGCTGAACGGCAGTGCTTACTCCAGCTCGGATCGGGATGACATG
<i>vn (probe 1)</i>	TAAAACAGCCAGATTTATGTGCCTGCCCTGTGGGATTGCATT
<i>vn (probe 2)</i>	GGCTTATTCGATGCAAAGGTGCCGTCTGCCAACAGCTCTGGCTTCCATATATCGGACATCTTGAATTTGGAGGGCTCTGAGCTGAAGAATGCAGCAGCTG
<i>vnd</i>	AATATCTTAAGTAGCTGCTCAGCCTCAACAGCATCTTAGTAGCCGTAGCAATGTTAAGTTAAAGCCAGCAACGATTCGACGCCGCGCAGCAGTA
<i>yl</i>	GGCTTATTCGATGCAAAAGGTGCCGTCTGCCAACGCTCTGGCTTCCATATATCGGACATCTTGAATTTGGAGGGCTCTGAGCTGAAGAATGCAGCAGCTG
<i>zen</i>	AGTTTCAGCCAGAGT
<i>zld/vfl</i>	TGAGATTGCTCAGCGCTTGTCCCTGTGCGAACGCCAGGTGAAGATCTGGTTCCAGAACCGACGAATGAAGTTCAAGAAGGACATACAAGGTCAACCGCGAG
<i>Rm62</i>	GGCACTGATTACGCCTTTGGCACCGTG
<i>dap</i>	TTTGGGCAACTACATCCAGATCAACATTGGATCGCTGGAGCTGTCCGCCAATCACAACATCCGCCAGGTGGTGGACGTTTGTGACGAGTTTACAGCAAGGAG
<i>lys</i>	AATTAGTCAATTGCGGGACCGCGTGGAAGAATGGGGCAGGAAGCTGTTAAATTGGCAGAACGAACACCACATTTTGACCTTGTAGGGGCCATAGACCATAC
<i>phe</i>	TATGCGAGCAAAAGCATTCTCATCAGTCGCAATGATT
<i>thr</i>	CAGCAAGGTTCGTAGTGTGTTAACAGGAAACGGACTGAAAGATCCGAACACAGCGGTGACATTTT

Table S3. Transcript counts for all *Drosophila* genes included in the NanoString code set.

Gene	NC10	NC11	NC12	NC13 early	NC13 late	NC14A	NC14B	NC14C	NC14D	Gastrulation	twi-NC13 late	twi- NC14C	twi-Gastrulation
ASPP	8288	6688	7916	8155	10422	112335	450505	551935	876282	563944			
CIAPIN1	761129	542467	830513	734745	661469	467461	823911	695686	956214	430563			
Dl	935709	663601	518982	585214	888970	875290	1660717	1731491	2372444	1801880			
Doc1	37712	24320	35864	53056	48315	75013	152344	183487	214242	125614			
Egfr	16083	17321	61660	592139	669584	1868709	2109015	1957941	3003563	1421675			
LacZ	182186	293310	583454	232979	129289	547485	316991	409355	525149	287243			
Msh	7162	8986	11232	11402	15824	22560	50759	109471	165301	258317			
N	1558777	1189694	1111618	1211288	1546959	1167607	995446	1132822	1686271	866623			
NetA	6724	3591	18350	50983	123138	217278	752457	978334	996502	565661	96717	269647	382261
NetA	7842	2870	4869	4856	21973	60994	415134	430662	900211	324375	5090	124160	226021
Neu3	435331	139034	157481	56727	52197	27079	431172	724790	870291	934613			
Race	5129	7538	22196	100307	95235	245341	634014	531354	575584	549055			
RpL32	16905105	13720549	23781430	20722641	13362584	15668506	20545028	19085664	16268679	15827045			
RpL7-like	1326432	787879	753764	1092939	999343	938951	1615813	1393553	1402098	1120626			
SoxN	5309	5502	5098	15390	60970	220887	455703	664135	1123229	816014			
Su(H)	862571	572156	518462	847601	819821	1018784	1253770	1195990	1094373	1053576			
Tl	4389119	2970065	2936472	3842825	3701868	2677429	3675885	2532735	2410680	601151			
Wnt8	9774	6597	20135	21129	29529	9309	52747	191934	180746	248468			
YFP	11384	16091	54591	92940	9106	35942	37433	97376	52526	170385			
argos	31026	14868	15948	18819	25707	13444	168035	184339	180897	107737			
bcd	824064	593817	383500	972066	547987	490086	26033	10922	6458	5122			
brk	8398	5245	8674	32586	97685	344800	354206	422148	645661	283417			
cic	957247	673918	497843	436273	781548	612413	403992	360468	435226	263707			
dhd	79674243	21449661	14059531	8572801	2660381	1685924	595044	307924	486971	235065			
dpp	8632	14373	23964	86638	204534	304993	257196	187936	191752	119736			
eve	45060	103559	335322	458662	358758	810429	2109262	1718719	1451811	1093716			
fdy	8190983	2562347	2854680	1258724	814592	273225	763763	881542	809285	950438			
grh	5160	7656	6322	12892	46858	166102	982027	1135535	1440205	731692			
hb	1914781	955723	745552	1349177	1716938	2389012	1003582	713784	640024	332298			
hnt	23844	15592	18270	19535	20618	24522	43901	160272	247744	220214			
htl	13707	8416	9961	16187	36252	31042	424855	578598	746791	456443	15257	18281	284297
if	7483	5567	9669	9148	13287	8248	126433	250528	374144	242609			
ind	9782	6833	12585	13634	29097	9643	123908	242090	184619	180276			
m8	49895	30608	36453	119173	85588	61094	77560	148526	226797	147635			
mad	2320229	1606187	1499279	2120802	1992640	1775663	1566217	955707	1015417	364573			
med	769012	515610	744110	710495	585845	483242	514247	441612	410031	351410			
mes3	14145	13770	69304	229645	442062	712089	1663073	1633260	1616914	1138062			
otd	7397	4204	9937	65295	177593	479657	813160	746499	1064261	537093			
phm	2799	2375	7769	12665	47528	114560	651684	956336	792661	647817			
pnr	2627	1837	2797	6243	17618	11523	87999	202723	270344	373276			
pnt	247152	208096	282070	215594	179415	34791	156893	189810	235059	399086			
put	1225353	744442	525653	773866	956389	954800	706486	580462	542102	253371			
pyr	3800	5535	11320	9898	16944	36753	220649	230789	360600	188092			
rho	13199	6920	8970	23837	31396	167800	143865	130785	174815	64487			
sax	327269	236816	405846	361637	213050	156841	134686	124414	154724	104473			
scw	48284	110085	434630	913014	925065	422228	162527	73058	76205	12681			
shn	480283	298802	218485	313696	389072	641353	717375	658500	915969	370918			
sim	19353	11501	17189	11473	10200	13827	23298	60815	124627	145286			
sli	41021	29031	29068	109052	208056	557530	1890349	2128538	3005397	1938660			
sna	48743	39533	80456	174096	416652	481341	1196169	1442597	1341323	967958	473406	274218	527574
sog	8108	7109	7152	81861	160970	699008	2203870	1668058	2108290	1548549			
sog	23511	26939	163448	193414	147083	888861	1483394	897275	1235709	797801			
spi	301687	171465	141912	113561	174344	174985	1330121	1741162	2377326	1828567			
spz	1774940	1582678	3340007	2240101	829977	190455	73095	52781	70276	53562			
star	1494056	686253	536176	631415	756432	748682	775022	844008	1107001	775543			
stumps	8053	3612	3017	7669	7195	10694	540227	714826	848024	843642			
ths	5129	5136	5727	9809	18330	22865	270168	359123	663613	517465			
tin	5012	4259	7211	4327	15468	4852	24035	81743	117617	234121			
tkv	798740	487457	401224	561750	606528	539503	641227	481711	728126	512325			
tld	71046	126383	287898	661702	636138	1197603	1004434	827969	818131	319511			
tsg	78961	184946	956782	874300	657930	740006	723239	503869	412145	197618			
tup	3542	3689	7538	17393	49082	80574	580765	702873	714659	510458			
twi	13573	20122	116034	370665	557544	1164521	3974135	3884520	3257041	2854478	325489	944528	1187181
ush	82242	27256	29118	15126	43747	15505	561491	842846	977968	907121			
vn	26053	22694	14342	31297	25129	29354	96505	81081	87258	74668			
vn	6779	8620	10006	15198	23184	99396	145492	108087	201069	137041			
vnd	10048	4296	8142	18357	68824	191621	267890	306959	406459	209536			
yl	11487866	5404336	5176009	5078951	1382270	410441	190539	134058	146212	94954			
zen	117039	190098	444769	1375704	1091425	2617444	389283	341477	311618	194090			
zld	5522624	4260426	5020075	5357893	5900820	6979035	8844677	9350096	9438153	5305075			
Rm62	7297964	5060832	9263082	7311270	5693838	3719954	6563911	6556429	5546910	4898260			