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Report

Zelda Potentiates Morphogen Activity by Increasing Chromatin Accessibility

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Summary

Zygotic genome activation (ZGA) is a major genome programming event whereby the cells of the embryo begin to adopt specified fates. Experiments in *Drosophila* and zebrafish have revealed that ZGA depends on transcription factors that provide large-scale control of gene expression by direct and specific binding to gene regulatory sequences [1–5]. Zelda (Zld) plays such a role in the *Drosophila* embryo, where it has been shown to control the action of patterning signals [1, 2]; however, the mechanisms underlying this effect remain largely unclear. A recent model proposed that Zld binding sites act as quantitative regulators of the spatiotemporal expression of genes activated by Dorsal (Dl), the morphogen that patterns the dorsoventral axis [6]. Here we tested this model experimentally, using enhancers of *brinker* (*brk*) and *short gastrulation* (*sog*), both of which are directly activated by Dl, but at different concentration thresholds [7–9]. In agreement with the model, we show that there is a clear positive correlation between the number of Zld binding sites and the spatial domain of enhancer activity. Likewise, the timing of expression could be advanced or delayed. We present evidence that Zld facilitates binding of Dl to regulatory DNA, and that this is associated with increased chromatin accessibility. Importantly, the change in chromatin accessibility is strongly correlated with the change in Zld binding, but not Dl. We propose that the ability of genome activators to facilitate readout of transcriptional input is key to widespread transcriptional induction during ZGA.

Results and Discussion

In blastoderm embryos, *brinker* (*brk*) is activated in an eight- to ten-cell-wide domain that develops into the ventral neurogenic ectoderm (NE), whereas *short gastrulation* (*sog*) is expressed in a broader band of 16–18 cells encompassing the entire NE (see Figures 1A and 1I). Both genes have the same ventral expression boundary due to repression by Snail (Sna) in the presumptive mesoderm [11–15]. The dorsal borders of their domains lie in regions of the Dorsal (Dl) gradient where amounts are low and change little, raising the question of how their enhancers can interpret small differences in Dl concentrations.

sog and *brk* each have two reported *cis*-regulatory modules (enhancers) that are active in early embryos [10, 16–20]. The *sog* intronic lateral stripe enhancer (LSE) [16] is less well conserved and drives a slightly narrower stripe of expression relative to the *sog* shadow enhancer [17], also known as the neurogenic ectoderm enhancer (NEE), which recapitulates the broad endogenous *sog* pattern [18]. The *brk* 5′ and 3′ enhancers both support lateral stripes similar to endogenous *brk* [10, 17]; however, the *brk* 3′ enhancer drives a more dynamic pattern that broadens at cellularization [19]. Thus, we focused on the *brk* 5′ enhancer to avoid confounding dynamic change of width.

The *sog* 426 bp NEE contains three CAGGTAG heptamer sites for optimal Zelda (Zld) binding. However, the *brk* 498 bp 5′ enhancer does not have any canonical Zld binding sites (also known as TAGteam sites [21]). To explain its Zld dependence, we used electrophoretic mobility shift assays to look for Zld binding sites in the *brk* 5′ enhancer. We identified three CAGGTCA sequences and a tandem GAGGCAC AGGCAC sequence that promote very weak Zld binding, which was abolished upon mutation of the sites (see Figure S1 available online).

To test whether altering the number of Zld binding sites in the NE enhancers can affect the expression they drive, we created mutant forms of the *brk* and *sog* enhancers. The *sog* NEE (sog wt, Figure 1C) drives a *lacZ* reporter expression pattern identical to endogenous *sog* (Figure 1A). Mutation of all three CAGGTAG sites dramatically reduced the expression width (sog 0; Figures 1E and 1R). Similar changes were also observed by Liberman et al. (2009) when they mutated the CAGGTAG sites in the *sog* LSE [20]. Costaining of *lacZ* and endogenous *sog* illustrates that the narrowed *lacZ* domain resulted from a collapse of the dorsal, not the ventral, border (data not shown). We infer that without Zld, *sog* is unable to be activated by the lower levels of Dl in the dorsal neuroectoderm region. In embryos lacking maternal Zld [1] (referred to herein as *zld*$^{-}$), both the endogenous *sog* and sog wt domains shrink and become sporadic (Figures 1B and 1D). This is not due to an indirect effect on the Dl concentration gradient because it is unchanged in *zld*$^{-}$ (Figure S2). Thus, loss of Zld in *trans*, or Zld binding sites in *cis*, has the same effect on NEE activity, indicating a direct modulation of *sog* by Zld.

Next we performed the opposite experiment by introducing three CAGGTAG sites into the *brk* 5′ enhancer. This modified enhancer (brk +3a) drives a considerably expanded expression domain (Figure 1M) compared to brk wt (Figures 1K and 1R). A second form of the *brk* enhancer with CAGGTAG sites added to different locations (brk +3b) also drives the same expanded expression domain (Figure S3), arguing against the requirement of precise motif grammar in Zld's regulation of NE genes.

To rule out the possibility that the expansion in domain width of brk +3a is caused by inadvertent disruption of a repressor binding site rather than addition of Zld binding sites, we mutated the three added CAGGTAG sequences in brk +3a into 7-mers that are neither the original sequence nor Zld binding sites (Figure 1O; brk +3m). Mutation of these sites reduced the expanded domain of brk +3a back to a width similar to brk wt (Figure 1R). When each of the brk +3a, brk +3b, and brk +3m transgenic enhancers was placed into a *zld*$^{-}$ background, narrow and sporadic expression resulted

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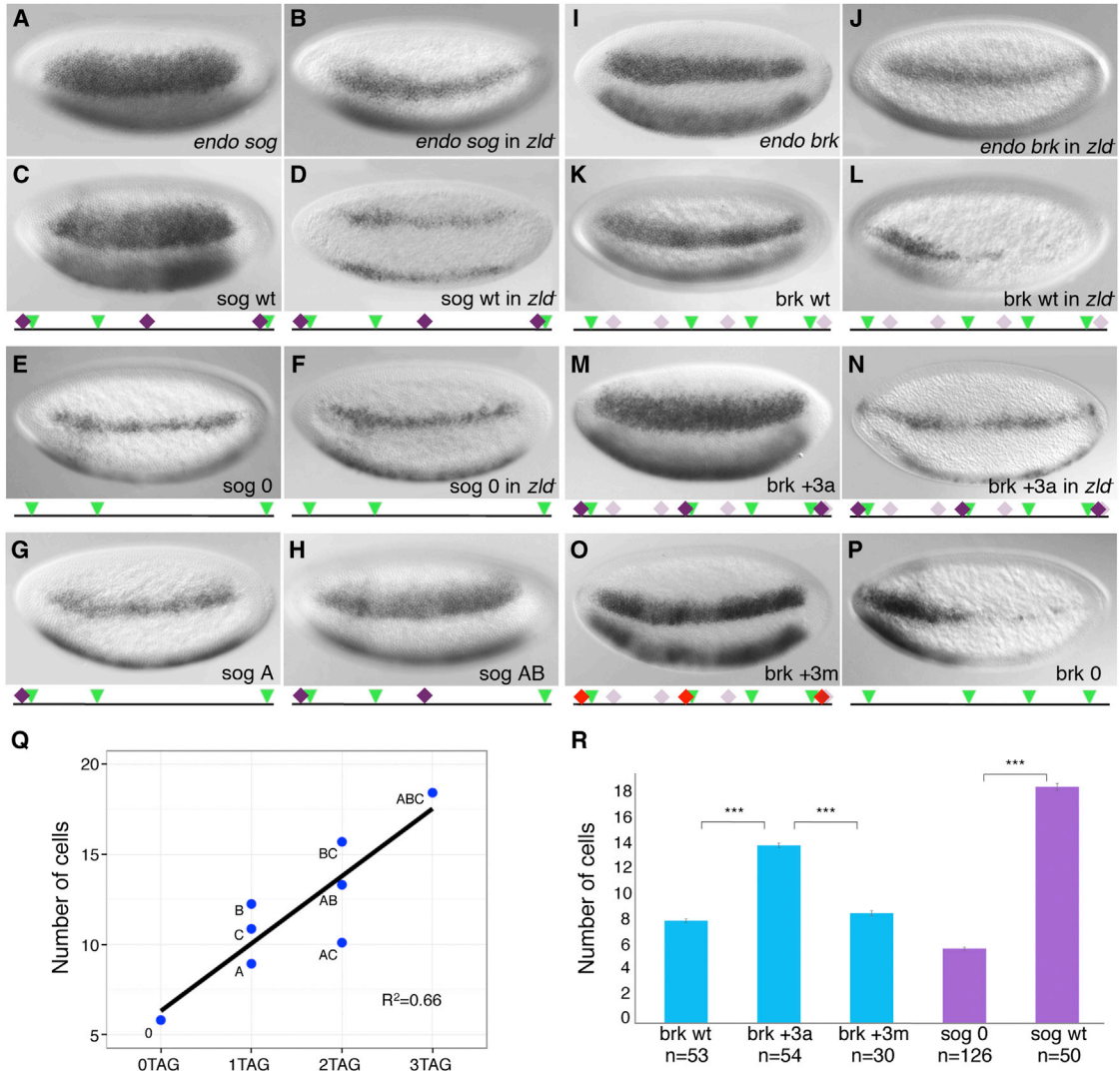


Figure 1. The Number of Zld Binding Sites Determines the Spatial Extent of Dl Target Gene Expression

Wild-type (A, C, E, G, H, I, K, M, O, and P) and *zld*$^{-}$ (B, D, F, J, L, and N) embryos in nuclear cycle (nc) 14 were hybridized with RNA probes synthesized against cDNA sequences for *sog* (A and B), *brk* (I and J), or *lacZ* (C–H and K–P) for transgenic embryos. Here and in subsequent figures, embryos are oriented anterior to the left and dorsal up. A schematic representation of the enhancer that drives *lacZ* expression is shown below transgenic embryos (C–H and K–P). Green triangle, Dl site; dark purple diamond, canonical Zld site; light purple diamond, noncanonical Zld site; red diamond, mutagenized Zld site.

(C–F) Mutation of all three Zld sites in the *sog* NEE caused a reduction in the expression domain it drives.

(G and H) Elimination of one (H) or two (G) Zld sites in *sog* NEE resulted in a stepwise narrowing of the expression domain.

(K–N) Addition of three Zld sites to the *brk* 5′ enhancer led to a Zld-dependent expansion in expression.

(O) Mutation of the three added Zld sites yielded an expression similar to that driven by the brk wt enhancer.

(P) Removal of all Zld sites in the *brk* 5′ enhancer led to sporadic and thin expression pattern. Anterior-posterior modulation seems to be in play for the expression of *brk*, which could be explained by the presence of two Bicoid (Bcd) sites in this enhancer [10].

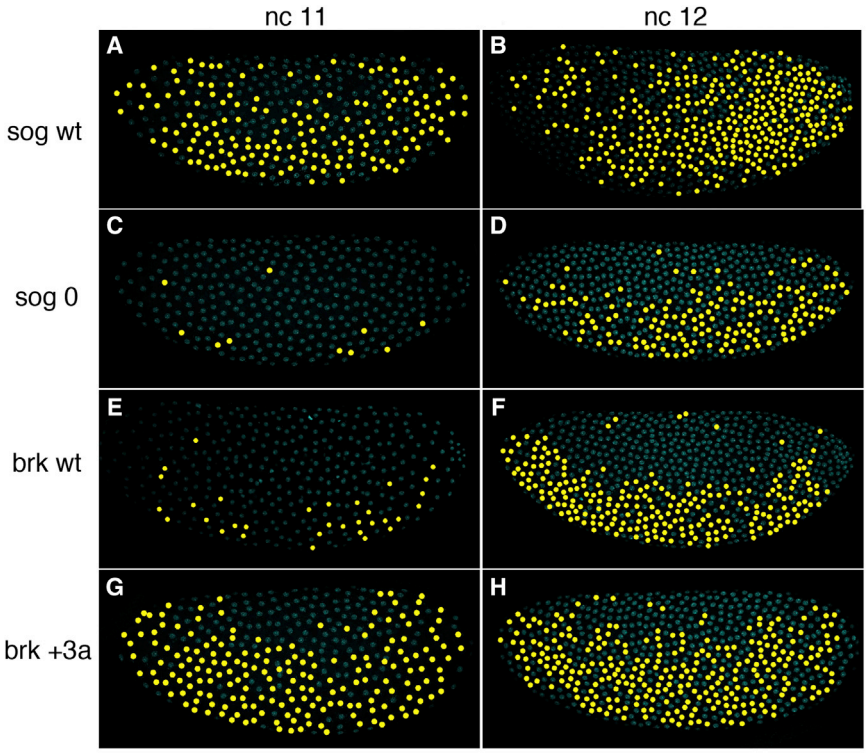
(Q) Scatterplot showing the width of expression domain (in the number of cells it spans) driven by different forms of the *sog* NEE that contain zero (0TAG), one (1TAG), two (2TAG), or three (3TAG) Zld sites. Each dot represents the average from at least 20 embryos. The width of expression domain correlates with number of Zld sites (linear regression $R^2 = 0.66$).

(R) Bar chart showing the width of expression domain driven by the brk wt, brk +3a, brk +3m, sog 0, and sog wt enhancers. Data are represented as mean ± SEM. ***$p < 0.005$, t test.

See also Figures S1–S3.

resembling that of endogenous *brk* in *zld*$^{-}$ (Figures 1J and 1N; data not shown), again supporting that the CAGGTAG-driven broadened expression is Zld dependent. Moreover, mutation of the newly found weak Zld binding sites led to a narrowed and weakened stripe of expression, identical to the pattern of brk wt in *zld*$^{-}$ (Figures 1L and 1P).

To better correlate the number of Zld sites with the extent of reporter expression, we constructed six different forms of the *sog* NEE containing either one or two of the three CAGGTAG sites (see Figure 1G for a one-site line [sog A] and Figure 1H for a two-site line [sog AB]). The width of expression correlated moderately to the number of Zld sites in the enhancer (Figure 1Q; $R^2 = 0.66$). However, some sites appear to be more important than others in contributing to the expression width, indicating a context dependency for Zld binding sites. From our results and others' work demonstrating weakened NE



I	sog wt	sog 0	brk wt	brk +3a
nc 9	0/5	0/5	0/7	0/7
nc 10	9/13	0/11	0/8	7/10
nc 11	14/15	6/13	6/13	9/10
nc 12	14/14	15/15	10/12	12/12

Figure 2. The Number of Zld Binding Sites Determines the Timing of Dl Target Gene Activation

nc 11 (A, C, E, and G) and nc 12 (B, D, F, and H) embryos carrying *sog* (A–D) or *brk* (E–H) transgenes were hybridized with RNA probes synthesized against intronic sequences of the *yellow* reporter gene. Dl antibody staining (not shown) was used to orient embryos. DAPI-stained nuclei expressing *yellow* reporter gene are pseudocolored in yellow. Compared to the sog wt enhancer (A and B), mutation of all Zld sites in the *sog* NEE (sog 0; C and D) results in delayed and sporadic expression.

(E–H) Embryos carrying the *brk* enhancer with added Zld sites (brk +3a; G and H) have advanced initiation of transcription compared to embryos carrying the brk wt enhancer (brk wt; E and F).

(I) Table showing the number of embryos carrying the four transgenic lines that display any expression from nc 9 to nc 12.

gene expression upon removal of Zld or Zld sites [1, 2, 20, 22, 23], it is evident that Zld is indispensable for the proper expression of NE genes.

We next asked whether the number of Zld binding sites also influences the timing of Dl target expression, since previous reports have implicated Zld as a developmental timer. Harrison et al. (2011) observed a correlation between the onset of zygotic gene expression and strength of Zld binding at nc 8 [3]. Besides that, when the enhancer region of *zen*, which contains four Zld binding sites, was multimerized, it drove precocious activation of reporter expression [21]. And finally, Nien et al. (2011) showed that the expression of many patterning genes is delayed in *zld*$^{-}$ embryos [2], including *sog* and *brk*. We reasoned that since Dl nuclear concentrations increase from nc 10 to nc 14 [24–26], the lower levels of Dl present in earlier cycles would no longer be adequate to activate target genes without Zld's input, resulting in delayed activation of *sog* and *brk* [6, 27].

To measure the onset of transcription, we determined when the four transgenic enhancers (sog wt, sog 0, brk wt, and brk +3a) could activate an intron-containing *yellow* reporter gene [28], which allows us to detect nascent transcripts. Reporter expression driven by the sog wt enhancer was first detectable in nc 10 embryos, whereas no reporter activity was observed for the sog 0 enhancer until nc 11 (Figure 2I; see false color of fluorescence in situ hybridization signal). Even in nc 12, the expression driven by sog 0 is more sporadic compared to sog wt (Figures 2A–2D). Unlike in nc 14 embryos, reporter expression can be seen in ventral nuclei of nc 11 and nc 12 embryos because the Sna repressor has not yet accumulated to high levels [15]. Adding three Zld sites to the *brk* enhancer resulted in advanced initiation of reporter activity from nc 11 to nc 10 (Figure 2I), and reporter expression also became more robust, in terms of both the proportion of nuclei showing expression and the ratio of embryos with expressing nuclei (Figures 2E–2I). Our results clearly illustrate that by manipulating Zld binding sites, the timing of NE gene activation can be altered. Temporal regulation by transcription factor binding sites has also been shown in *Ciona* where the number of Brachyury binding sites governs the timing of notochord gene expression [29].

We believe that Zld regulates the temporal and spatial expression of NE genes by promoting Dl activity, rather than acting independently, because nuclear Dl is absolutely required for the activation of *brk* and *sog*, which exhibit no expression in genetic backgrounds lacking nuclear Dl [20, 30]. One possible mechanism may involve cooperativity at the level of DNA binding [6]. To test the hypothesis that the extent of Zld binding impacts Dl binding at target enhancers, we performed chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) to measure Zld and Dl binding to the different transgenic enhancers.

The sog 0 enhancer without Zld sites has diminished Zld binding when compared to sog wt (t test, p = 0.004; Figure 3A). Dl binding is also much reduced (p = 0.002; Figure 3B). As an internal control, Zld and Dl binding to the endogenous *sog* locus showed no significant difference between the lines (p = 0.464 and 0.288, respectively; Figures 3A and 3B). On the other hand, introduction of Zld sites into the *brk* transgenic enhancer led to higher Zld binding (p = 0.0047; Figure 3D) and Dl binding (p = 0.004; Figure 3E), while Zld and Dl binding to the endogenous locus remained similar between lines (p = 0.221 and 0.452, respectively; Figures 3D and 3E). These results illustrate that changing the number of Zld sites, and therefore changing

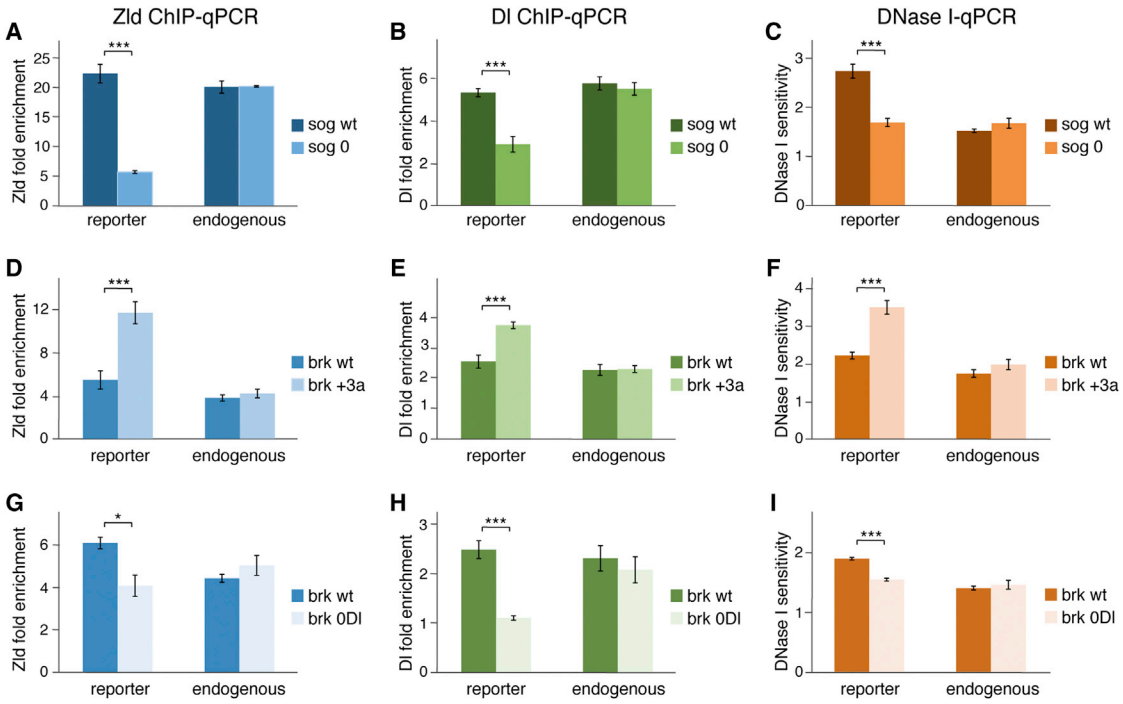


Figure 3. Zld Promotes Dl Binding to Target Enhancers and Increases Chromatin Accessibility

Bar charts showing Zld (A, D, and G) and Dl (B, E, and H) ChIP-qPCR results and DNase I digestion-qPCR results (C, F, and I) performed on 1.5–3 hr embryos carrying transgenic enhancers. Error bars indicate SEM from three biological replicates. *p < 0.05; **p < 0.01; ***p < 0.005.

(A–C) Embryos carrying the *sog* enhancer with mutated Zld sites (sog 0) have reduced Zld (A) and Dl (B) binding, as well as lower sensitivity to DNase I digestion (C), on the reporter region compared to embryos carrying the sog wt enhancer.

(D–F) The *brk* transgenic enhancer with added Zld sites (brk +3a) has higher Zld (D) and Dl (E) binding and higher sensitivity to DNase I digestion (F) than the brk wt enhancer.

(G–I) The *brk* transgenic enhancer with mutated Dl sites (brk 0Dl) has reduced Zld (G) and Dl (H) binding and slightly lower sensitivity to DNase I digestion (I) than the brk wt enhancer. Shown are ChIP enrichment or DNase I hypersensitivity relative to an unrelated genomic region (see Experimental Procedures).

the amount of Zld binding to the NE enhancers, influences the level of Dl binding to its target sites in vivo.

Our results from reporter expression analyses and ChIP experiments suggest that Zld promotes transcriptional output by facilitating Dl DNA binding. Zld might directly interact with Dl, leading to cooperative DNA binding as in the Dl-Twist (Twi) interaction [14, 31–33]. Alternatively, Zld might assist factor binding by interacting with common coactivators or by changing the local chromatin accessibility [34, 35]. We favor the latter possibility for several reasons: (1) Zld binding greatly overlaps with that of many other transcription factors such as Bcd, Hunchback, Dl, Twi, Sna, and Mothers against Dpp (Mad) [2]; (2) Zld helps the binding of Twi and Bcd to target DNA [23, 36]; (3) the presence of Zld binding sites is associated with high levels of transcription factor binding [37]; and (4) the Zld site (CAGGTA; [2]) is the most enriched motif in transcription factor binding "HOT regions," which were seen to correlate with decreased nucleosome density [37–39]. Hence, it is more likely that Zld plays a more general role, such as "opening" the underlying chromatin, than that it interacts specifically with multiple other factors.

We therefore went on to address the hypothesis that Zld facilitates the binding of Dl by making the local chromatin more accessible. DNase I's preferential digestion of nucleosome-depleted DNA in the genome can be used to map active regulatory regions accessible for transcription factor binding [40, 41]. We performed DNase I hypersensitivity assays followed by qPCR (DNase I-qPCR) to measure the chromatin "openness" of transgenic enhancers carrying varying numbers of Zld sites. The *sog* transgenic enhancer region had significant reduction of chromatin accessibility when Zld sites were mutated (~1.6-fold, p = 0.002; Figure 3C), while adding Zld sites to the *brk* transgenic enhancer increased sensitivity to DNase I digestion (~1.6-fold, p = 0.002; Figure 3F). The DNase I hypersensitivity assessed on endogenous *brk* and *sog* loci were comparable between transgenic lines (p = 0.118 and 0.114, respectively; Figures 3C and 3F), serving as a control for embryo staging between transgenic lines and the DNase I digestion procedure.

These results suggest that the presence of Zld sites, and thus Zld binding, makes the local chromatin more accessible for Dl, and potentially other transcription factors. However, it is feasible that the total number of factor binding sites influences chromatin accessibility rather than the number of Zld sites in particular. Therefore, we assayed the DNase I hypersensitivity of a transgenic *brk* enhancer that lacks all Dl binding sites and shows no reporter expression (brk 0Dl; Figure S3). Dl binding decreased nearly to background levels (~2.3-fold, p = 0.001; Figure 3H) compared to brk wt, but the Zld binding and DNase I hypersensitivity showed only slight decreases (~1.5-fold, p = 0.012 and ~1.2-fold, p = 0.0002, respectively; Figures 3G and 3I), which is not comparable to the effects seen upon manipulation of Zld sites on the *brk* and *sog* enhancers (Figures 3A–3F). We reason that the binding of each transcription factor may contribute to the DNase I hypersensitivity to a certain extent but that the major influence comes from Zld

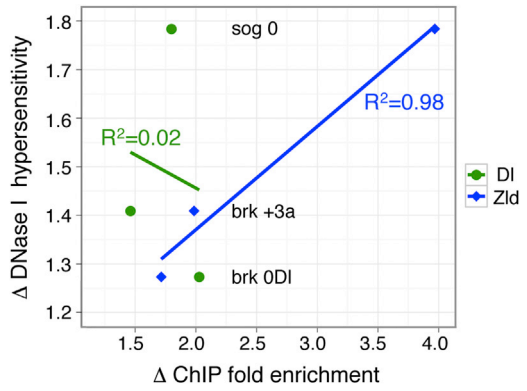


Figure 4. The Change in Chromatin Accessibility Correlates with the Change in Zld Binding on Target Enhancers

Zld and Dl ChIP enrichment and DNase I hypersensitivity on the transgenic region were normalized to the endogenous enhancer locus, and the fold change was then calculated for the two lines under comparison (sog 0 versus sog wt, brk +3a versus brk wt, and brk 0Dl versus brk wt). Blue dots and green dots represent Zld and Dl, respectively. The change in Zld binding between lines strongly correlates with the change in DNase I hypersensitivity (linear regression R^2 = 0.98), whereas the change in Dl binding does not (R^2 = 0.02).

binding. To further evaluate the contribution of Zld versus Dl sites to chromatin accessibility, we calculated the fold change in Zld and Dl binding for sog 0, brk +3a, and brk 0Dl relative to their corresponding wt transgenic enhancers and then correlated the fold change in factor binding with the change in DNase I hypersensitivity (Figure 4). We found a strong correlation between the change in Zld binding and DNase I hypersensitivity (R^2 = 0.98), whereas the change in Dl binding and DNase I hypersensitivity do not correlate (R^2 = 0.02). These results support the idea that the number of Zld sites rather than Dl sites is important in determining chromatin accessibility.

Using Zld's coregulation of NE genes as a case in point, we have shed light on how Zld functions as a zygotic genome activator. Our data reveal that Zld works in combination with Dl and regulates Dl target genes by binding differentially to their regulatory sequences. Changing the number of Zld sites on Dl target gene enhancers has a pronounced effect on their expression both temporally and spatially. As a uniformly distributed factor, Zld supplies positional information by promoting Dl binding to target enhancers, thereby increasing the "apparent dosage" of Dl. Zld's input is especially important where the level of morphogen is low and likely plays a similar role for other key factors in the blastoderm embryo, such as Twi, Bcd, and Mad. Uniform factors have been found to act in combination with Sonic Hedgehog in neural tube differentiation [42], and our findings on how Zld potentiates morphogen activity will be relevant to vertebrate systems.

Although our results do not rule out other possible mechanisms, they strongly support the idea that Zld binding increases chromatin accessibility, which we believe contributes greatly to how it activates such a wide range of targets. In this model, the amount of Zld binding on a region would determine how open and therefore how active it is. At the center of this property is Zld's ability to occupy a large fraction of its recognition sites in early embryos [3]. Besides that, Zld is present in nuclei as early as nc 2 [2], which is considerably earlier than other factors (e.g., Bcd, nc 6; Dl, nc 10) [25, 26, 43]. Therefore, Zld may act as a pioneer factor as previously suggested [3, 27], but whether Zld binds to its sites in nucleosomes and repositions them, or whether it recruits histone modifiers that in turn affect binding of other factors like Dl, awaits further investigation. Interestingly, this idea may extend beyond flies, since newly discovered genome activators in zebrafish zygotic genome activation have been seen to cooperate with developmental regulators and prime the genome for subsequent activation [4, 5]. Thus, it seems that developmental control of zygotic genome activation is highly similar in flies and fish.

Experimental Procedures

Transgenic Reporter Analysis

Mutant forms of the 426 bp *sog* NEE and the 498 bp *brk* 5′ enhancer were created via site-specific mutagenesis or by direct synthesis using Integrated DNA Technologies custom gene synthesis service. Enhancer and primer sequences can be found in Supplemental Experimental Procedures. In situ hybridization and antibody staining were performed as described previously [1, 44].

Zld and Dl ChIP-qPCR

ChIP was performed on 1.5–3 hr embryos using a modified protocol from the Zeitlinger lab [45]. Three biological replicates were performed for each ChIP experiment. Three primer sets (see Supplemental Experimental Procedures for primer sequences) were used to probe the reporter locus ($target_{Out}$), the endogenous enhancer ($target_{En}$), and an unrelated genomic region on chr2L (control), respectively. ChIP enrichment was then calculated as ($ChIP_{target}$/$ChIP_{control}$)/($input_{target}$/$input_{control}$) for both reporter and endogenous loci.

DNase I Digestion-qPCR

DNase I digestion was performed on 1.5–3 hr embryos as described previously [41], with some modifications. Three biological replicates were performed for each DNase I digestion experiment. The same primer sets as in the ChIP-qPCR experiments were used. We first calculated the percent remaining DNA at target loci relative to the control region, which did not show DNase I hypersensitivity [41], and then normalized the percent remaining DNA after 15 min digestion to that without DNase I digestion, giving rise to normalized percent remaining DNA ([$target_{15min}$/$control_{15min}$]/[$target_{0min}$/$control_{0min}$]). DNase I hypersensitivity was finally presented as 1/(normalized percent remaining DNA) for both reporter and endogenous loci.

Supplemental Information

Supplemental Information includes three figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.04.032.

Author Contributions

S.M.F., Y.S., N.K., S.Y.S., and C.A.R. conceived and designed the experiments. S.M.F., Y.S., B.L., R.Z., K.O., and N.K. performed the experiments. All authors analyzed the data. S.M.F., Y.S., S.Y.S., and C.A.R. wrote the paper.

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