Regulation of *Ubx* Expression by Epigenetic Enhancer Silencing in Response to *Ubx* Levels and Genetic Variation

Michael A. Crickmore¹*, Vikram Ranade², Richard S. Mann³*

¹Department of Biological Sciences, Columbia University, New York, New York, United States of America, ²Department of Genetics and Development, Columbia University, New York, New York, United States of America, ³Department of Biochemistry and Molecular Biophysics, Columbia University, New York, New York, United States of America

**Abstract**

For gene products that must be present in cells at defined concentrations, expression levels must be tightly controlled to ensure robustness against environmental, genetic, and developmental noise. By studying the regulation of the concentration-sensitive *Drosophila melanogaster* Hox gene *Ultrabithorax* (*Ubx*), we found that *Ubx* enhancer activities respond to both increases in *Ubx* levels and genetic background. Large, transient increases in *Ubx* levels are capable of silencing all enhancer input into *Ubx* transcription, resulting in the complete silencing of this gene. Small increases in *Ubx* levels, brought about by duplications of the *Ubx* locus, cause sporadic silencing of subsets of *Ubx* enhancers. *Ubx* enhancer silencing can also be induced by outcrossing laboratory stocks to *D. melanogaster* strains established from wild flies from around the world. These results suggest that enhancer activities are not rigidly determined, but instead are sensitive to genetic background. Together, these findings suggest that enhancer silencing may be used to maintain gene product levels within the correct range in response to natural genetic variation.

**Introduction**

The transcriptional control of gene expression in eukaryotes is governed by cis-regulatory elements, also known as enhancers, that integrate cell-type and temporal information by binding combinations of transcription factors. Genes that exhibit complex expression patterns are typically controlled by multiple cis-regulatory elements, some of which have overlapping, partially redundant activities [1,2,3,4]. Current estimates suggest that from 10 to 80% of the non-coding DNA of higher eukaryotes is devoted to gene regulation [5,6,7], raising the question of how all of this regulatory information is integrated to generate accurate and stereotyped patterns of gene expression in space and time. A third dimension of gene regulation is quantity, which is especially relevant for genes that must be expressed within a narrow range of levels. One possible solution is that enhancers are precisely tuned to generate the appropriate level of transcription that is required in each cell. However, the precision that this type of mechanism demands seems difficult to achieve and especially vulnerable to genetic, environmental, and developmental noise. An alternative solution is that feedback or other regulatory mechanisms exist that modulate enhancer activities in response to the levels of gene product. Although feedback autoregulation is a well-known motif in transcriptional networks [8], mechanisms that might be used to tune expression levels are not well understood. This problem is particularly challenging for genes that have multiple, partially redundant regulatory inputs.

We have begun to study this problem in the fruit fly, *Drosophila melanogaster*, by analyzing the mechanisms that control the expression of the Hox gene *Ultrabithorax* (*Ubx*) in the haltere—a dorsal appendage on the third thoracic segment (T3) that helps the fly balance during flight [9]. Although Ubx protein is detected in all cells of the developing haltere imaginal disc, its pattern of expression is not uniform [10] (Figure 1A). Subsets of the complex regulatory input into the *Ubx* locus can be monitored by examining the expression patterns of *Ubx* enhancer traps, which exhibit different, overlapping patterns of expression (Figure 1). For example, *Ubx-Gal4^{R5dom}* is expressed uniformly throughout the anterior (A) compartment of the haltere disc, but only in the distal portion of the posterior (P) compartment (Figure 1B). In contrast, *Ubx-Gal4^{R5dom}* is expressed in distal regions in both the A and P compartments but is not expressed proximally (Figure 1D).

**Results/Discussion**

*Ubx* negative autoregulation

Somewhat paradoxically, transient ectopic expression of Ubx, induced either by heat shock or Gal4-mediated expression, resulted in *Ubx* loss-of-function transformations that can be visualized both in the adult (as haltere to wing transformations; [11]) and in 3rd instar haltere imaginal discs (as groups of cells that showed a reduction or complete loss of Ubx protein) [12]...
Author Summary

Gene expression is generally governed by cis-regulatory elements, also called enhancers. For genes whose expression levels must be tightly controlled, enhancer activities must be tightly regulated. In this work, we show that enhancers that control the expression of the Hox gene Ultrabithorax (Ubx) in Drosophila are regulated by a negative autoregulatory feedback mechanism. Negative autoregulation can be triggered by less than a two-fold increase in Ubx levels or by varying the genetic background. Together, these data reveal that enhancer activities are not always hardwired, but instead may be sensitive to genetic and environmental variation and, in some cases, to the amount of gene product they regulate. The finding that enhancers are sensitive to genetic background suggests that the regulation of gene expression is more plastic than previously thought and has important implications for how transcription is controlled in vivo.

(Figure 2). Thus, a transient pulse of high Ubx protein levels can lead to the complete and inheritable silencing of all Ubx expression, implying that Ubx is being silenced by its own gene product.

Transient pulses of ectopic Ubx also resulted in the stable silencing of Ubx enhancer traps, including Ubx-Gal4lac1, Ubx-Gal4M1, Ubx-Gal4DN, and Ubx-lacZ^166 (Figure 2 and Table S1). When the absence of Ubx protein was observed, these cells also had no enhancer trap expression (Figure 2). However, in many cases enhancer trap silencing was observed in cells that had normal Ubx protein levels (Figure 2). In these cases we suggest that only the enhancers captured by the enhancer trap were silenced, and that other, partially redundant, enhancers in the Ubx locus remained active, resulting in an apparently normal pattern of Ubx expression. We also find, consistent with previous results [12], that the patches of Ubx-silenced cells in the haltere are clonal events and that the Polycomb system of epigenetic regulators is required for silencing (Figure S1 and Figure S2).

To obtain initial mechanistic insights into Ubx autoregulatory silencing, we carried out experiments that suggest it requires specific DNA binding by Ubx. For these experiments, we monitored the ability of chimeric Hox proteins to induce haltere-to-wing transformations when expressed via the vg-Gal4 driver. Although the more anterior Hox protein Antennapedia (Antp) was unable to induce Ubx silencing, transient overexpression of Antp-Ubx chimeric proteins revealed that the Ubx homeodomain and adjacent C-terminal sequences were both necessary and together sufficient to induce robust Ubx silencing (Figure 3). These findings suggest that Ubx protein, and not Ubx mRNA, is responsible for the induction of silencing. Further, as both the homeodomain and adjacent sequences are implicated in Ubx specificity and DNA binding [13,14,15], these results suggest that Ubx triggers silencing...
by binding to Ubx-specific cis-regulatory elements. Consistently, the Hox protein Abd-A (Abd-A), which is very similar to Ubx in both domains, also induced Ubx silencing when transiently expressed during haltere development (Figure 3).

**Ubx enhancer silencing triggered by additional copies of the Ubx+ gene**

We next tested whether more subtle increases in Ubx levels could also induce silencing. For these experiments, we monitored the expression of Ubx lacZ or Gal4 enhancer traps in flies that had extra copies of the wild type Ubx locus. Ubx-Gal4<sup>HS</sup> and Ubx-Gal4<sup>LDN</sup> were silenced in groups of haltere cells of 3x Ubx<sup>+ </sup>and 4x Ubx<sup>+ </sup>flies (100% of 4x Ubx<sup>+ </sup>haltere discs had at least one group of silenced cells) (Figure 4A–4D; Table S1). In these haltere discs, probably because the flies had multiple copies of Ubx<sup>+</sup>, the pattern of Ubx protein was invariably wild type (Figure 4A, 4B, 4D). Interestingly, the amount of silencing induced by 4 copies of Ubx was significantly decreased when one of these copies encoded a non-functional Ubx protein (the Ubx<sup>9-22</sup> allele; data not shown). This result supports the idea that Ubx protein, not Ubx mRNA, is

---

**Figure 2. Ubx enhancer silencing in response to hs-Ubx.** (A) Wild type haltere disc stained for Ubx protein. Note the higher levels in the distal region. (B) Haltere disc in which an HA-tagged Ubx protein was expressed via the vg-Gal4 driver, which is transiently expressed in all haltere cells. The disc was stained for HA (green) and Ubx (red). At this stage, the vg-Gal4 driver is active along the dorso-ventral boundary (strong green and yellow stain). Groups of cells that do not stain for Ubx (arrow) are observed. (C) Adult haltere from a vg>Ubx fly showing a transformation from haltere to wing. Both wing margin (arrow) and wing blade (arrowhead) tissue is observed. (D,E) Ubx-Gal4<sup>MT</sup> (D) and Ubx-Gal4<sup>LDN</sup> (E) haltere discs that were given a transient pulse of Ubx expression by heat shock during the 2nd instar, stained for GFP (green, to monitor enhancer trap activities) and Ubx (red). Some cells no longer express the enhancer traps and Ubx (arrows). Some cells no longer express the enhancer traps, but still express Ubx (arrowheads). (F) Wild type Ubx-Gal4<sup>ON</sup> haltere disc stained for GFP (green, to monitor the enhancer trap) and Ubx (red). (G) A Ubx-Gal4<sup>ON</sup> haltere disc that was given a transient pulse of Ubx expression by heat shock during the 2nd instar, stained for Ubx (red) and GFP (green, to monitor the enhancer trap). Silencing of both Ubx and the enhancer trap are observed (arrows). Surrounding the Ubx silenced cells, some cells have reduced Ubx levels but still express the enhancer trap.

doi:10.1371/journal.pgen.1000633.g002
Haltere size and Ubx levels are buffered in response to increased Ubx+ copy number

The above results show that epigenetic autoregulatory silencing of Ubx enhances occurs in response to elevated Ubx levels. Interestingly, increasing the dose of Ubx+ results in smaller halteres [16], but this size change does not scale linearly with the number of Ubx+ genes. Haltere size is similar to wild type in flies with 3x Ubx+ or 4x Ubx+, while in flies with 6 copies of Ubx+, haltere size is greatly reduced (Figure 5A and Figure S4A). These results suggest that haltere size is buffered against increasing doses of the Ubx gene. A similar buffering can be observed when Ubx protein levels are quantified in haltere discs from animals with different numbers of Ubx+ genes. When one copy of Ubx is inactivated (1x Ubx+), Ubx protein levels are nearly halved (Figure S4A). However, when the Ubx+ complement is doubled (4x Ubx+) or tripled (6x Ubx+) only 39% and 60% increases in Ubx protein levels were detected, respectively (Figure S4A). The less-than-expected increases in Ubx levels seen in Ubx duplications is not because they fail to express wild type levels, as they are sufficient to fully rescue a Ubx null mutation, both phenotypically [17,18] and with respect to Ubx protein levels (data not shown).

Together with the results described above, we suggest that the buffering of Ubx levels and haltere size is due, at least in part, to the epigenetic silencing of Ubx enhancers in response to higher than normal doses of Ubx+.

Ubx enhancer silencing induced by genetic variation

In wild type animals, we hypothesized that enhancer silencing may be used to ensure uniform Ubx levels in response to naturally occurring genetic variation in the cis- and trans-regulation of Ubx expression. We tested this idea by out-crossing our laboratory Ubx-Gal4 F1 flies to 32 D. melanogaster strains established from wild
populations around the world. In our lab stock, less than 5% of haltere discs showed any evidence of Ubx-Gal4lac1 silencing. However, when outcrossed to wild D. melanogaster strains, we frequently observed silencing of Ubx-Gal4lac1 in haltere discs of the F1 generations (Figure 5 and Table S2). Although the frequency of silencing varied between wild stocks, it was consistent for each wild stock in a statistically significant manner (Figure 6). Of the 32 stocks crossed to Ubx-Gal4lac1, 14 resulted in no detectable silencing in the F1 generation, 6 showed weak silencing in the F1 generation, and 12 showed strong silencing in the F1 generation.

Figure 4. Ubx enhancer trap silencing in response to increasing Ubx+ dose. (A) Ubx-Gal4lac1 is silenced in groups of cells by 4 copies of the Ubx+ locus (arrows), but Ubx protein levels are normal. (B) Ubx-Gal4lac1 is silenced in groups of cells by 3 copies of the Ubx+ locus (arrows), but Ubx protein levels are normal. (C) Wild type haltere expression pattern of Ubx-Gal4LDN. (D) Ubx-Gal4LDN is partially silenced by 4 copies of the Ubx+ locus. (E-G) Wild type haltere expression patterns of Ubx-lacZlac1 (E), Ubx-Gal4M1 (F), and Ubx-Gal4M3 (G). (H-J) Ubx-lacZlac1 (H) and Ubx-Gal4M1 (I), but not Ubx-Gal4M3 (J), are partially silenced by 4 copies of Ubx+. Note that for Ubx-lacZlac1 and Ubx-Gal4M1, silencing does not occur in random clones, but instead is manifest by a loss of expression in proximal regions of the disc (arrows).

doi:10.1371/journal.pgen.1000633.g004
Figure 5. *Ubx* enhancer silencing in response to natural genetic variation. (A) Halteres decrease in size with increasing *Ubx* copy number. 

*Bxd(1)Df(1)/+ (1xUbx+); Wild Type (2xUbx+); Dp(1)P/+(3xUbx+); Dp(1)P/+(4xUbx+); Dp(1)P/+(5xUbx+); Dp(1)P/+(6xUbx+). (B–U) All images show haltere discs stained for enhancer trap expression. 

(B–Q) *Ubx-Gal4* driven UAS-GFP reporter expression in the lab stock (B) and outcrossed to various wild type stocks (C–Q). Stocks beginning with "NC2" were collected in North Carolina. Other wild type stocks were obtained from the Bloomington Stock Center. See Table S1 and Table S2 for a complete summary of these results. (C–H) Outcrossing to these stocks does not cause *Ubx-Gal4* silencing. (I–L) Outcrossing to these wild type stocks causes mild to moderate *Ubx-Gal4* silencing. (M–Q) Outcrossing to these wild type stocks causes moderate to strong *Ubx-Gal4* silencing. (R,S) *Ubx-Gal4* in the lab background (R) and in F1 progeny when crossed to Tw2 (S). Strong clonal silencing is observed. (T,U) *Ubx-Gal4* in the lab background (T) and in F1 progeny when crossed to NC2-76 (U). Loss of proximal expression (arrows) is observed.

doi:10.1371/journal.pgen.1000633.g005
% Silencing

Figure 6. Quantification of silencing. Each point records the % silencing of the Ubx-Gal4Δ40 enhancer trap for a single haltere disc. % silencing is defined as the amount of non-stained tissue relative to wild type controls measured in parallel (see Materials and Methods for details). Unless otherwise indicated, all measurements were of haltere discs from F1 animals grown under non-crowded conditions produced by crossing our laboratory Ubx-Gal4Δ40 stock to the indicated genetic backgrounds (4× Ubx, orange circles; NC2-80, black triangles; NC2-76, pink diamonds; Ber2, green squares; Tw2, blue triangles; Harwich, tan circles). Silencing was measured in two independent sets of crosses, separated in time by two weeks, and are graphed in neighboring columns. The thick black bars correspond to averages and the thinner bars show the standard error of the mean. For each cross, a minimum of 10 haltere discs from 10 different animals, were scored. An analysis of variance (ANOVA) shows that the differences among the five wild type genotypes (NC2-80, NC2-76, Ber2, Tw2 and Harwich) in % silencing were highly significant (t ratio = 9.4, p = 0.0007) with 83% of the variance among lines, and no differences between replicates. Also graphed is the % silencing measured in 10 independent haltere discs resulting from the continued introgression (F3 generations) of Ubx-Gal4Δ40 into the NC2-80 background (black circles) and into the NC2-76 background (pink circles). The average % silencing increased in the F3 generations compared to the F1 generations. doi:10.1371/journal.pgen.1000633.g006

Plasticity of enhancer activities

In the crosses to wild D. melanogaster strains, when kept in isolation from each other, may have subtly different ways of regulating Ubx. These may be due to strain-specific differences in the Ubx cis-regulatory elements, in the trans regulators of Ubx expression, or both. Consistent with this idea, it is of interest that gene expression levels, when assayed across entire genomes, show a lot of variability in natural populations [21,22,23,24,25]. Although we find that the final Ubx expression pattern and levels are very similar between lab and wild D. melanogaster strains, when two strains are bred together genetic differences may result in fluctuations in the initial Ubx levels. The silencing system described here may function to compensate for these fluctuations and thus ensure that the correct Ubx levels are produced throughout the haltere.

Enhancer silencing and natural genetic variation

Most remarkably, we found that enhancer silencing can occur simply by varying the genetic background. In Drosophila melanogaster, due in part to its large population size, the frequency of DNA polymorphisms between individuals in the wild is estimated to be as high as 1 in 100 basepairs [20]. Due to these polymorphisms, we imagine that different strains of D. melanogaster, when kept in isolation from each other, may have subtly different ways of regulating Ubx. These may be due to strain-specific differences in the Ubx cis-regulatory elements, in the trans regulators of Ubx expression, or both. Consistent with this idea, it is of interest that gene expression levels, when assayed across entire genomes, show a lot of variability in natural populations [21,22,23,24,25]. Although we find that the final Ubx expression pattern and levels are very similar between lab and wild D. melanogaster strains, when two strains are bred together genetic differences may result in fluctuations in the initial Ubx levels. The silencing system described here may function to compensate for these fluctuations and thus ensure that the correct Ubx levels are produced throughout the haltere.

Enhancer silencing at Ubx

We postulate that silencing induced in these outcrosses may be due to an incompatibility between the trans-acting factors (largely derived from the wild stocks) and cis-regulatory elements (linked to the monitored Ubx locus of the laboratory stock) controlling Ubx expression. In support of this idea, when Ubx-Gal4Δ40 was further introgressed into weakly or strongly silencing wild stocks, which effectively increases the genetic complement from the wild strain background, an increase in the severity of silencing was observed when compared to the F1 generation (Figure 6 and Figure S5). We also never observed the complete absence of Ubx protein or haltere-to-wing transformations in any of these outcrosses, arguing that only a subset of enhancer inputs into Ubx is silenced in response to genetic variation. Consistently, individual enhancer traps responded differently when crossed to the same wild strains (Table S1).

Together, these results demonstrate that Ubx enhancer silencing is triggered when Ubx is present at higher than normal levels. When Ubx concentration is especially high (when Ubx is ectopically expressed via Gal4 or heat-shock promoters) all enhancer input into Ubx can be silenced, resulting in the complete absence of Ubx expression and haltere-to-wing transformations. Although such high levels of Ubx are not physiological, we also find that Ubx enhancer silencing can be triggered by additional copies of Ubx+, which in principle results in less than double the amount of Ubx protein. In this case, we find that the expression of some Ubx enhancer traps is clonally silenced (e.g. Ubx-Gal4Δ40), while the expression of other enhancer traps (e.g. Ubx-lacZ) is reduced. Thus, different Ubx enhancers are differentially sensitive to negative autoregulation; some are shut off by relatively low Ubx levels, while others require high Ubx levels to be silenced.
outputs, regardless of genetic background. Instead, due to natural genetic variation, the activity of a particular enhancer may vary widely between individuals in wild populations. Additionally, our results show that the activity of an enhancer can even vary among the cells within its expression domain (e.g. the haltere) in a single individual. We suggest that plasticity in enhancer activities is essential to compensate for genetic and perhaps environmental variation. Moreover, given that many genes may have multiple, partially redundant enhancers, enhancer silencing may be essential to buffer gene expression levels so that they remain within a narrow, biologically tolerable range. On the other hand, small differences in enhancer activities in flies in the wild may serve as a potential source of phenotypic variation that can be acted upon by natural selection. Since population genetic theory predicts that selection differentials of a small fraction of a percent are seen in natural populations with the effective population size of Drosophila [20], it is plausible that this variation is functionally significant, perhaps through a subtle influence of haltere morphology on flight performance.

Materials and Methods

Genetic variation experiments

The NC2 stocks were obtained from Greg Gibson (N.C. State University); all other wild stocks were obtained from the Bloomington Stock Center (Table S2).

To show that the lack of expression in these outcrosses was not due to a failure to initiate enhancer trap expression in the wild backgrounds, we carried out a lineage tracing experiment. The genotype of the stock was: Ubx-Gal4lac1 UAS-flp; actin>stop>GFP. The combination of UAS-flp and actin>stop>GFP records the history (i.e. marks the lineage) of Gal4 expression. When outcrossed to wild backgrounds, GFP expression was not silenced (in contrast to when the direct UAS-GFP readout was monitored). Together, these results suggest that Ubx-Gal4lac1 was initially activated but then silenced.

Hybrid dysgenesis was ruled out as a reason for loss of expression from P transposons by the following tests: 1) silencing occurs equally well, regardless of the direction the cross was set up, 2) silencing occurs equally well at 18° and 25°C (while hybrid dysgenesis is suppressed at 18°C), 3) silencing was not observed for some other transposon insertions (inside or outside of the Ubx locus) when crossed to the same wild stocks, 4) the miniwhite gene associated with the P element insertions did not lead to a variegated eye phenotype as would be expected for somatic dysgenesis, and 5) quantitative PCR analysis confirmed that the amount of transposon DNA was the same in the parent (unsilenced) and F2 (silenced) generations. Finally, enhancer trap expression can be recovered when back-crossed into the laboratory stock background.

Quantification of Ubx protein levels

To measure Ubx protein levels in different genetic backgrounds, we stained haltere discs obtained from uncrowded yw (2x Ubx+), yw; If/Cyo;TM2/TM6B (1x Ubx+), yw; If/Cyo;DpP5/TM6B (3x Ubx+), yw; DpP10x2/CyoGFP; MKRS/TM6B (4x Ubx+), yw; DpP10x2/CyoGFP; DpP5/TM6B (6x Ubx+), Hikone-R, Berlin-K, NC2-76, NC2-80, yw x NC2-76 F1s, yw x Tw-2 F1s, Florida-9, Reids-2, and Harwich wandering larvae with anti-Ubx (FP3.38) and a fluorescent secondary antibody. Stainings and confocal imaging were done identically and in parallel for ≥8 haltere discs from each genotype. The pixel intensities in identically sized regions of the distal anterior compartments were measured using Adobe Photoshop. This region was quantified because it is a relatively large area that expresses Ubx at uniform levels and gives rise to the main body of the haltere (the same portion measured in Figure 3A and Figure S4A). Similar trends were observed when average pixel intensities for the entire distal haltere were measured. The average intensities for each wild population differed by no more than 16%, suggesting that final Ubx levels are very similar despite differences in genetic background and silencing.

Quantification of Ubx reporter silencing

To quantify the extent of silencing of the Ubx-Gal4lac1 reporter in response to Ubx+ copy number and outcrosses to wild populations, third instar haltere discs were dissected from wandering larvae of yw 122; DpP10x2/CyoGFP; Ubx-Gal4lac1 UAS-GFP/TM6B (4x Ubx+), and the GFP positive, F1 progeny of yw 122; If/Cyo; Ubx-Gal4lac1 UAS-GFP/TM6B crossed with NC2-80, NC2-76, Ber-2, Tw-2, and Harwich. GFP positive F3 progeny of yw 122; If/Cyo; Ubx-Gal4lac1 UAS-GFP/TM6B crossed with NC2-80 and NC2-76 were also dissected. For the outcrosses, we always used females from the wild populations. Haltere discs were fixed, mounted, and imaged for GFP and DAPI on a confocal microscope. Images were made binary in ImageJ. The GFP expressing area relative to the total disc area was measured for each disc, and this value was subtracted from the average GFP expressing area (relative to total disc size) of yw 122; If/Cyo; Ubx-Gal4lac1 UAS-GFP/TM6B haltere discs to yield a “% silencing” value for each disc.

Heat-shock induced Ubx overexpression

Larvae bearing the hi-Ubxla22 transgene [26] were heat-shocked at 37°C for 15–20 minutes 3 or 4 days after egg laying. Larvae were dissected at least 48 hours after heat shock to allow for total dissipation of exogenous Ubx. hi-Ubxla22 larvae that were not heat shocked showed no Ubx silencing. Neutral clones were induced using the same heat shock regime in flies of the genotype yw hsflp; FRT 42D Ubx-GFP/FRT 42D; hs-Ubxla22/+.

Ubx enhancer traps and duplications

Ubx-Gal4lac1 [27]; Ubx-lacZ166 [28]; Ubx-Gal4loxP [29]; Ubx-Gal4loxP [29]; Ubx-lacZ166 [30]; and Ubx-Gal4loxP [29]. Although these lines are hypomorphic mutations of the Ubx locus, this is unlikely to contribute to our results because decreased production of Ubx would, if anything, cause an underestimate of the amount of silencing that occurs at the Ubx locus.

3x Ubx+ flies contain a tandem duplication of the Ubx locus (Dp3;3P5).

4x Ubx+ flies contain a tandem duplication of a transposition of the Ubx locus onto the 2nd chromosome (Dp3;2P10). Further increases in Ubx+ copy number were created by combining these duplications [16]. Ubx+ flies expresses a non-functional Ubx protein due to a ~1500 bp deletion that removes a splice acceptor site and part of the Ubx homeodomain-encoding exon [31].

Before crossing to enhancer traps, Ubx duplications were introduced into stocks containing marked chromosomes that do not cause silencing, yw hsflp; If/cyo; DpP5/TM6B and yw hsflp; Dp3;2P10x2/CyoGFP; MKRS/TM6B.

To monitor silencing of Ubx-lacZ166 and Ubx-Gal4lac1 simultaneously (Figure S3), flies of the genotype, Dp3;2P10x2/heat shock: Ubx; Ubx-lacZ166/loxP Ubx-Gal4loxP UAS-GFP were given a 15 min. heat shock at 37°C 48 to 96 hrs after egg laying. Imaginal discs were dissected at wandering stage and stained for Ubx, βgal, and GFP. Silencing was not observed in flies of the same genotype without heat shock.
Antp-Ubx chimeras

(Previously described by [14]
UAS-Antp
UAS-AUA
UAS-UU* (* refers to a stop codon inserted immediately following the homeodomain)
UAS-AU
UAS-AU

Quantitative PCR

Whole-fly genomic DNA was isolated from the lab stock containing the Ubx-Gal4F0 enhancer trap [yw122; if/CyoGFP; Ubx-Gal4F0] UAS-GFP/TM6B and the GFP+ F2 progeny of the Ubx-Gal4F0 stock crossed to strains Tw2, NC2-76, and NC2-80. Silencing was confirmed to be occurring in these crosses. The F2 progeny were generated by crossing Gal4F0 UAS-GFP F1 males to wild population females, precluding the possibility of recombination between chromosomes of the lab and wild genotypes. Primers were designed to amplify ~200 bp in the Gal4 and UAS transgenes to determine their relative abundance in each genotype. A ~200 bp sequence in the 5’UTR of homothorax was amplified to normalize for different amounts of template DNA. PCR amplification was performed in triplicate using Applied Biosystems 7300 Real Time PCR System, and SYBR Green PCR Master Mix. Product dissociation curves were examined to ensure that each primer set only amplified a single product. Ct values and amplification curves were consistent with an equal abundance of the Gal4 and UAS sequences in all genotypes.

Antibody staining

Standard protocols were used with the following primary antibodies:
Rabbit anti-β-Gal 1:10,000 (Cappel)
Mouse anti-En 1:10 (Hybridoma Bank)
Mouse anti-Ubx 1:20
Rat anti-HA 1:100

Supporting Information

Figure S1 Neutral clones respect the borders of Ubx silencing. (A,B) Two examples of haltere discs with neutral clones (marked by the absence of GFP) and Ubx silencing (induced by hs-Ubx). In (A), there is no crossing between the neutral clones and Ubx-silenced patches. In (B), although most of the neutral clones respect the Ubx-silenced patches, there are two small exceptions (arrows). Ubx- silenced patches are outlined in yellow and the neutral clones are outlined in blue. The exceptions observed in these experiments are likely due to multiple neutral clones that were scored as a single clone because they fused during growth.

Found at: doi:10.1371/journal.pgen.1000633.s001 (9.78 MB TIF)

Figure S2 PcG functions are required for Ubx autoregulatory silencing. (A) Wing disc with PcI clones (absence of GFP) stained for Ubx (red) and GFP. Ubx expression is observed in pock clones. (B) Haltere disc with PcI clones (absence of GFP) stained for Ubx (red) and GFP. Ubx expression is unaffected by the absence of PcI. Pcl was the only PcG gene we tested to show strong, autonomous Ubx derepression in the wing disc, and no affect on Ubx expression in the haltere disc; the PcG mutations Pe, Soc, ph, and Su(Z)2 could not be used for this experiment because they result in a loss of Ubx expression in the haltere, due to the derepression of more posterior Hox genes (data not shown). (C) A Ubx-Gal4F0+ haltere disc in which both silencing (by hs-Ubx) and PcI clones were induced. PcI- tissue is outlined in yellow. Silencing of both Ubx and the enhancer trap are observed, but not in PcI- tissue. Note that a PcI clone only affect Ubx expression in the distal, “pouch” domain of the wing and haltere (Beuchle D, Struhl G, Muller J (2001) Polycomb group proteins and heritable silencing of Drosophila Hox genes. Development 128: 993-1004).

Found at: doi:10.1371/journal.pgen.1000633.s002 (7.73 MB TIF)

Figure S3 Simultaneous monitoring of silencing for two Ubx enhancer traps. (A,B) hs-Ubx/DpP10x2; UbxGal4Galo1 UAS-GFP/ UbxlacZ166 haltere disc from animals that were not given a heat shock (A) or were given a 15 min heat shock (B). The discs were stained for Ubx (blue), GFP (green), and βgal (red). Individual channels are shown as indicated. For (B), where silencing was observed, the outlines of the silenced clones are shown as follows: in the βgal channel (B’) the outlines of Ubx (yellow outline) silenced clones are shown. In the GFP channel (B’) the outlines of Ubx (yellow outline) silenced clones are shown. B’ shows the GFP channel with the Ubx-lacZ166 (red outline) silenced clones. Note that the extent of silencing of Ubx-Gal4F0 is greater than that of Ubx-lacZ166, and that Ubx-lacZ166 silencing is a subset of Ubx-Gal4F0 silencing.

Found at: doi:10.1371/journal.pgen.1000633.s003 (3.13 MB TIF)

Figure S4 Quantification of haltere sizes and Ubx levels. (A) Quantifications of Ubx protein levels (blue bars) and haltere sizes (red bars) in genotypes with differing numbers of wild type Ubx+ alleles. Both measurements are shown relative to wild type (2x Ubx+). Note that neither measurement scales quantitatively with increases in Ubx+ dose, illustrating that these phenotypes are buffered. In contrast, one copy of Ubx+ shows a ~60% reduction in Ubx protein levels and a ~50% increase in haltere size compared to wild type (2x Ubx+). Error bars represent standard error of the mean. (B) Quantifications of Ubx levels in 8 different wild genetic backgrounds (Hikone-R, Berlin-K, NC2-80, NC2-76, Tw2, Florida-9, Reids-2, and Harwich) and two F1s (yw X NC2-76 and yw X Tw2) are all within ~16% of those measured in yw. Moreover, this variation does not correlate with the degree of silencing (shown in the thumbnail images below the graph). For
comparison, halving the dose of Ubx+ decreases Ubx levels by ~40% (left-most bar). Error bars represent standard error of the mean.

Found at: doi:10.1371/journal.pgen.1000633.s004 (0.36 MB TIF)

Figure S5 Ubx silencing increases with introgression into wild genetic backgrounds. (A) Ubx-Gal4lac1 expression in the F1 progeny of a cross to the Tw2 wild type line. (B) Silencing increases when Ubx-Gal4lac1 is introgressed by backcrossing into the Tw2 line. Shown here is a haltere disc after 2 backcrosses (the F3 generation). (C) Ubx-Gal4lac1 expression in the F1 progeny of a cross to the NC2-80 wild type line. (D) Silencing increases when Ubx-Gal4lac1 is introgressed by backcrossing into the NC2-80 line. Shown here is a haltere disc after 2 backcrosses (the F3 generation).

Found at: doi:10.1371/journal.pgen.1000633.s005 (1.47 MB TIF)

Table S1 Summary of Ubx enhancer traps and their responses to changes in Ubx levels and genetic variation

References

1. Yuh CH, Davidson EH (1996) Modular cis-regulatory organization of Endo16, a gut-specific gene of the sea urchin embryo. Development 122: 1069–1082.

2. Keys DN, Lee BI, Di Gregorio A, Harafuji N, Detter JC, et al. (2005) A saturation screen for cis-acting regulatory DNA in the Hox genes of Ciona intestinalis. Proc Natl Acad Sci U S A 102: 679–683.

3. Davidson EH (2001) Genomic Regulatory Systems. New York: Academic Press. 261 p.

4. Arnosti DN (2003) Analysis and function of transcriptional regulatory elements: insights from Drosophila. Annu Rev Entomol 48: 579–602.

5. Cameron RA, Oliveri P, Wyllie J, Davidson EH (2004) cis-Regulatory activity of randomly chosen genomic fragments from the sea urchin. Gene Expr Patterns 4: 205–213.

6. Birney E, Stamatoyannopoulos JA, Dutta A, Guigo R, Gingeras TR, et al. (2004) Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. Nature 447: 799–816.

7. Pfaffler B, Jenttt A, Hammonds A, Ngo T, Mira S, et al. (2008) Tools for Neuroanatomy and Neurogenetics in Drosophila. Proc Natl Acad Sci U S A In press.

8. Alon U (2007) Network motifs: theory and experimental approaches. Nat Rev Genet 8: 450–461.

9. Dickinson MH (1999) Haltere-mediated equilibrium reflexes of the fruit fly, Drosophila melanogaster. Philos Trans R Soc Lond B Biol Sci 354: 903–916.

10. White RA, Wilcox M (1984) Protein products of the bithorax complex in Drosophila. Cell 39: 163–171.

11. Irvine KD, Botas J, Jha S, Mann RS, Hogness DS (1993) Negative autoregulation by Ultrabithorax controls the level and pattern of its expression. Development 117: 387–399.

12. Garaulet DL, Foronda D, Calleja M, Sanchez-Herrero E (2008) Polycomb-dependent Ultrabithorax Hox gene silencing induced by high Ultrabithorax levels in Drosophila. Development 135: 3219–3229.

13. Chan SK, Mann RS (1993) The segment identity functions of Ultrabithorax are contained within its homeo domain and carboxy-terminal sequences. Genes Dev 7: 796–811.

14. Gehrlein B, Culi J, Ryoo HD, Zhang W, Mann RS (2002) Specificity of Distalless repression and limb primordia development by abdominal Hox proteins. Dev Cell 3: 487–498.

15. Merabet S, Saadou M, Sambrani N, Hudry B, Pradel J, et al. (2007) A unique Extradenticle recruitment mode in the Drosophila Hox protein Ultrabithorax. Proc Natl Acad Sci U S A 104: 16946–16951.

16. Snollik-Ulbaa SM (1999) Dosage requirements of Ultrabithorax and bithoraxoid in the determination of segment identity in Drosophila melanogaster. Genetics 124: 357–366.

17. Struhl G (1984) Splitting the bithorax complex of Drosophila. Nature 308: 454–457.

18. Lewis EB (1978) A gene complex controlling segmentation in Drosophila. Nature 276: 565–570.

19. Castro JP, Carazo CM (2004) Drosophila melanogaster: P transposable elements: mechanisms of transposition and regulation. Genetics 121: 107–118.

20. Aquadro CF, Bauer DuMont Y, Reed FA (2001) Genome-wide variation in the human and fruitfly: a comparison. Curr Opin Genet Dev 11: 627–634.

21. Schadt EE, Monks SA, Drake TA, Lusis AJ, Che N, et al. (2003) Genetics of gene expression surveyed in maize, mouse and man. Nature 422: 297–302.

22. Morley M, Molony CM, Weber TM, Devlin JB, Ewens KG, et al. (2004) Genetic analysis of genome-wide variation in human gene expression. Nature 430: 743–747.

23. Rockman MV, Kruglyak L (2006) Genetics of global gene expression. Nat Rev Genet 7: 862–872.

24. Ronald J, Bren RB, Whittle J, Kruglyak L (2005) Local regulatory variation in Saccharomyces cerevisiae. PLoS Genet 1: e25. doi:10.1371/journal.pgen.0010025.

25. Raser JM, O’Shea EK (2005) Noise in gene expression: origins, consequences, and control. Science 309: 2010–2013.

26. Mann RS, Hogness DS (1998) Functional dissection of Ultrabithorax proteins in D. melanogaster. Cell 60: 597–610.

27. Pallavi SK, Shashidhara LS (2003) Egr/Ras pathway mediates interactions between peripodial and disc proper cells in Drosophila wing discs. Development 130: 4931–4941.

28. Casares F, Bender W, Merriam J, Sanchez-Herrero E (1997) Interactions of Drosophila Ultrabithorax regulatory regions with native and foreign promoters. Genetics 145: 123–137.

29. de Nacar L, Foronda D, Suzanne M, Sanchez-Herrero E (2006) A simple and efficient method to identify replacements of P{lacZ} by P{Gal4} lines allows obtaining Gal4 insertions in the bithorax complex of Drosophila. Mech Dev 123: 860–862.

30. Bender W, Hudson A (2000) P element homing to the Drosophila bithorax complex. Development 127: 3981–3992.

31. Weinzirl RO, Axton JM, Glynis A, Akam ME (1987) Ultrabithorax mutations in constant and variable regions of the protein coding sequence. Genes Dev 1: 390–397.

Table S2 Summary of Ubx-Gal4lac1 silencing in F1 crosses to wild stocks

Found at: doi:10.1371/journal.pgen.1000633.s007 (0.07 MB DOC)

Acknowledgments

We thank G. Gibson, I. Dworkin, B. Gehrlein, T. Jessell, L. Johnston, and D. Rogulja for helpful discussions and/or comments on the manuscript. G. Gibson for carrying out the ANOVA analysis, and L. Vosshall for hosting MAC during the final stages of experiments. We thank E. Sanchez-Herrero, W. Bender, G. Gibson, and J. Muller for providing fly stocks.

Author Contributions

Conceived and designed the experiments: MAC VR RSM. Performed the experiments: MAC VR. Analyzed the data: MAC VR RSM. Wrote the paper: MAC VR RSM.