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Control of Hox transcription factor concentration and cell-to-cell variability by an auto-regulatory switch

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Key words

Auto-regulation, Fluorescence Correlation Spectroscopy, Hox genes, Protein noise, Transcription factors, Variability

Summary statement

Preferentially repressing and activating isoforms of the Hox transcription factor Antennapedia elicit a developmental regulatory switch from auto-activation to auto-repression which increases concentration and suppresses cell-to-cell variability over time.

Abstract

The variability in transcription factor concentration among cells is an important developmental determinant, yet how variability is controlled remains poorly understood. Studies of variability have focused predominantly on monitoring mRNA production noise. Little information exists about transcription factor protein variability, since this requires the use of quantitative methods with single-molecule sensitivity. Using Fluorescence Correlation Spectroscopy (FCS), we characterized the concentration and variability of 14 endogenously tagged TFs in live *Drosophila* imaginal discs. For the Hox TF Antennapedia we investigated whether protein variability results from random stochastic events or is developmentally regulated. We

43 found that Antennapedia transitioned from low concentration/high variability early, to
44 high concentration/low variability later, in development. FCS and temporally resolved
45 genetic studies uncovered that Antennapedia itself is necessary and sufficient to drive
46 a developmental regulatory switch from auto-activation to auto-repression, thereby
47 reducing variability. This switch is controlled by progressive changes in relative
48 concentrations of preferentially activating and repressing Antennapedia isoforms,
49 which bind chromatin with different affinities. Mathematical modelling demonstrated
50 that the experimentally supported auto-regulatory circuit can explain the increase of
51 Antennapedia concentration and suppression of variability over time.

52

53 **Introduction**

54 In order to understand the mechanisms that control pattern formation and cell
55 fate specification in developing organisms, the intranuclear concentration, DNA-
56 binding kinetics and cell-to-cell variability of relevant transcription factors (TFs) need
57 to be quantified. TF concentration variability at the tissue level is thought to arise from
58 diverse processes, including mRNA transcription, translation and protein degradation.
59 Intrinsic noise is due to stochastic binding and interactions of proteins involved in
60 transcriptional activation of a specific gene (Blake et al., 2003; Elowitz et al., 2002).
61 Extrinsic noise arises from inter-cellular differences in abundance of the transcriptional
62 and post-transcriptional machinery (Swain et al., 2002).

63 In undifferentiated tissue or cells, TF cell-to-cell variability can be the driving
64 force for differentiation. For example, progressive establishment of a Nanog salt-and-
65 pepper expression pattern leads to the formation of primitive endoderm in the mouse
66 preimplantation embryo, whereas loss of the variability results in embryos lacking
67 primitive endoderm entirely (Kang et al., 2013).

68 Conversely, in already differentiated tissue or cells, TF expression variability
69 among cells may need to be counteracted to ensure homogeneity of gene expression
70 patterns and robustness of commitment to a certain transcriptional regime. Examples
71 are the Snail (Sna) TF, which is required for the invagination of the mesoderm during
72 *Drosophila* gastrulation (Boettiger and Levine, 2013), or the Bicoid (Bcd) and
73 Hunchback (Hb) TFs during early embryogenesis (Gregor et al., 2007a; Gregor et al.,
74 2007b; Little et al., 2013).

75 In addition, differential cell fates within the same developmental territory may
76 be specified by TFs deploying different DNA-binding dynamics despite the existence
77 of very similar concentrations (i.e. low variability). For example, studies on the Oct4
78 TF in early mouse embryos have shown that differential kinetic behavior of DNA
79 binding, despite equal Oct4 concentration among blastomeres, ultimately dictates an
80 early developmental bias towards lineage segregation (Kaur et al., 2013; Plachta et
81 al., 2011).

82 So far, studies of gene expression variability have focused predominantly on
83 monitoring the noise of mRNA production (Holloway et al., 2011; Holloway and Spirov,
84 2015; Little et al., 2013; Lucas et al., 2013; Pare et al., 2009). Little information exists
85 about TF variability at the protein level within a tissue. Such studies require the use of
86 quantitative methods with single-molecule sensitivity.

87 We have previously used Fluorescence Correlation Spectroscopy (FCS), to
88 quantitatively characterize Hox TF interactions with nuclear DNA in living salivary
89 gland cells (Papadopoulos et al., 2015; Vukojevic et al., 2010). FCS is instrumental for
90 quantifying TF dynamics in living cells or tissue (Clark et al., 2016; Kaur et al., 2013;
91 Lam et al., 2012; Mistri et al., 2015; Papadopoulos et al., 2015; Perez-Camps et al.,
92 2016; Szaloki et al., 2015; Tiwari et al., 2013; Tsutsumi et al., 2016). However, in these

93 studies, mobility has only been measured for overexpressed proteins. However, to
94 understand TF behavior *in vivo*, proteins need to be quantified at endogenous levels
95 (Lo et al., 2015).

96 In this study, we take advantage of the availability of fly toolkits, in which TFs
97 have been endogenously tagged by different methodologies: fosmid (Baumgartner et
98 al., 1996), BAC (deposition of lines of Rebecca Spokony and Kevin White to Flybase
99 and the Bloomington Stock Center), FlyTrap (Buszczak et al., 2007; Kelso et al., 2004;
100 Morin et al., 2001; Quinones-Coello et al., 2007) and MiMIC lines (Nagarkar-Jaiswal
101 et al., 2015; Venken et al., 2011), to measure the intranuclear concentration of various
102 TFs *in vivo* by FCS, and their cell-to-cell variability in fly imaginal discs. Imaginal discs
103 are flat, single-layered epithelia comprised of small diploid cells and many TFs are
104 expressed in defined regions within these tissues during development.

105 106 **Results**

107 108 Characterization of average protein concentrations and cell-to-cell variability of 109 *Drosophila* TFs

110 Average concentrations of TFs in neighboring nuclei of third instar imaginal
111 discs were measured by FCS (Fig. 1A-J and Supplemental Fig. S1A-P). FCS is a non-
112 invasive method with single molecule sensitivity, in which a confocal arrangement of
113 optical elements is used to generate a small (sub-femtoliter) detection volume inside
114 living cells, from which fluorescence is being detected (Fig. 1C,D; green ellipsoid).
115 Fluorescent molecules diffuse through this observation volume, yielding fluorescence
116 intensity fluctuations that are recorded over time by detectors with single-photon
117 sensitivity (Fig. 1E). These fluctuations are subsequently subjected to temporal
118 autocorrelation analysis, yielding temporal autocorrelation curves (henceforth referred
119 to as FCS curves, Fig. 1F), which are then fitted with selected models to extract
120 quantitative information about the dynamic processes underlying the generation of the
121 recorded fluctuations. In the case of molecular movement of TFs (Supplement 1),
122 information can be obtained regarding: a) the absolute TF concentrations (Fig. 1F), (b)
123 TF dynamic properties, such as: diffusion times, differences in their interactions with
124 chromatin and fractions of free-diffusing *versus* chromatin-bound TFs (Fig. 1G); and
125 c) cell-to-cell TF concentration variability (Fig. 1H).

126 We selected 14 TF based on the availability of homozygous, endogenously
127 tagged transgenes and generation of robust fluorescence in distinct patterns in various
128 imaginal discs. For the 14 TFs, we measured average concentrations ranging about
129 two orders of magnitude among different TFs, from ~ 30 nM to ~ 1.1 μ M (~ 400 to 15500
130 molecules per nucleus, respectively) (Fig. 1I, Supplemental Fig. S1A-Q and
131 Supplement 2). Various diffusion times and fractions of slow and fast diffusing TF
132 molecules (Fig. 1J) indicated differential mobility and degree of DNA-binding among
133 different TFs (Vukojevic et al., 2010). Comparison of the y-axis amplitudes at the zero
134 lag time of the FCS curves, which are inversely proportional to the concentration of
135 fluorescent molecules (Fig. 1F), gives information about concentration variability
136 (heterogeneity) among different cell nuclei, i.e. reflects heterogeneity of protein
137 concentration at the tissue level (Fig. 1H). For all 14 TFs studied, the variability,
138 expressed as the variance over the mean squared, $CV^2 = \frac{s^2}{m^2}$, was determined to be
139 in the range 7 – 37% (Fig. 1K and Supplemental Fig. S1Q).

140 In biological systems, the Fano factor, expressed as the variance over the mean
141 ($F_f = \frac{s^2}{m}$, in concentration units), is a commonly used index to quantify variability. It has

142 been proposed that Fano factor values that increase with average concentrations
143 indicate that the underlying transcriptional processes cannot be sufficiently explained
144 by a simple one-step promoter configuration with purely intrinsic Poissonian noise and
145 that extrinsic noise is likely to contribute significantly to the overall variability (Newman
146 et al., 2006; Schwanhausser et al., 2011; Taniguchi et al., 2010). For all TFs
147 measured, Fano factor values from 0 to 20 were obtained (Supplemental Fig. S1R), in
148 line with Fano factor values of other TFs determined previously to lie between 0 and
149 30 (Sanchez et al., 2011). Moreover, the majority of TFs examined show Fano factor
150 values, $F_f > 1$, suggesting that transcriptional bursting is likely to be a significant
151 source of the observed cell-to-cell variability. ~~$F_f \approx 1$~~ ~~$F_f < 1$~~ We used this dataset as a
152 starting point for studying the control of variability during imaginal disc development.

153 The average concentration and variability of the investigated TFs showed no
154 obvious interdependence (Fig. 1K), suggesting that if variability is controlled, there is
155 not one control mechanism that is common to all investigated TFs. Among the studied
156 TFs, the Hox protein Antennapedia (Antp), showed low variability ($CV^2 < 0.2$) in high
157 average concentrations, in particular in the leg disc (Fig. 1K). Since low variability at
158 the tissue level is likely to be achieved through regulatory mechanisms, we
159 investigated Antp variability further by FCS. Because FCS performs best at low to
160 moderate expression levels (Supplement 1), we performed this analysis in the wing
161 disc where expression levels are lower than in the leg disc (Fig. 1K,L). We first
162 established that the observed fluorescence intensity fluctuations were caused by
163 diffusion of TF molecules through the detection confocal volume (Supplemental Fig.
164 S2 and Supplement 1). FCS showed that different clusters of neighboring cells along
165 the Antp expression domain in the wing disc display different average expression
166 levels (Fig. 1L). Moreover, FCS showed that Antp cell-to-cell variability decreased with
167 increasing Antp concentration (Fig. 1M) whereas the Fano factor increased
168 (Supplemental Fig. S1R). Such behavior is indicative of complex transcriptional
169 regulatory processes (Franz et al., 2011; Smolander et al., 2011) that we further
170 investigated using the powerful *Drosophila* genetic toolkit.

171

172 Control of Antp concentration by transcriptional auto-regulation

173 One mechanism by which genes control their expression level variability is
174 auto-regulation (Becskei and Serrano, 2000; Dublanche et al., 2006; Gronlund et al.,
175 2013; Nevozhay et al., 2009; Shimoga et al., 2013; Thattai and van Oudenaarden,
176 2001). To test whether Antp can regulate its own protein levels, we monitored the
177 concentration of endogenous Antp protein upon overexpression of *Antp* from a
178 transgene. To distinguish between overexpressed and endogenous protein, we used
179 synthetic Antp (SynthAntp) transgenes fused to eGFP (SynthAntp-eGFP). These
180 transgenes encode the Antp protein (amino acids 278-378), which includes the
181 homeodomain, the conserved YPWM motif and the C terminus (but lack the long and
182 non-conserved N terminus of the protein, against which widely used Antp antibodies
183 have been raised) and they harbor Antp-specific homeotic function (Papadopoulos et
184 al., 2011). Clonal overexpression of *SynthAntp-eGFP* in the wing disc notum (Fig.
185 2A,B',D and controls in Supplemental Fig. S3D,D') repressed the endogenous Antp
186 protein, indicating that Antp is indeed able to regulate its own protein levels.

187 Since Antp is a TF, we next asked whether the auto-repression occurs at the
188 transcriptional level. The *Antp* locus is subject to complex transcriptional regulation,
189 involving a distal and a proximal promoter (P1 and P2 promoters, respectively),
190 spanning more than 100 kb of regulatory sequences. We established that the P1
191 promoter (rather than the P2 promoter) is predominantly required to drive expression

192 of Antp in the wing disc notum (Supplemental Fig. S3A-C'), in line with previous
193 observations ((Engstrom et al., 1992; Jorgensen and Garber, 1987; Zink et al., 1991)
194 and Materials and Methods). Moreover, mitotic recombination experiments in regions
195 of the wing disc unique to P2 transcription have shown no function of the P2 promoter
196 transcripts in wing disc development (Abbott and Kaufman, 1986). Thus, the P1 Antp
197 reporter serves as a suitable reporter of the *Antp* locus transcriptional activity in this
198 context.

199 Clonal overexpression of SynthAntp-eGFP in the wing disc repressed the *Antp*
200 P1 transcriptional reporter (Fig. 2C-D and controls in Supplemental Fig. S3E,E'). To
201 rule out putative dominant negative activity of the small SynthAntp-eGFP peptide, we
202 also performed these experiments with the full-length Antp protein (Supplemental Fig.
203 S3F,F') and found them to also repress the reporter. We conclude that the Antp protein
204 is able to repress its own transcription from the P1 promoter (directly or indirectly),
205 suggesting a possible mechanism of suppressing cell-to-cell variability of *Antp*
206 expression levels (Fig. 2E).

207 In the course of these experiments, we noticed that ectopic overexpression of
208 *SynthAntp-eGFP* or the full-length Antp protein from the *Distal-less (Dll)* (MD23)
209 enhancer resulted in activation of the *Antp* P1 reporter in distal compartments of the
210 wing disc, such as the wing pouch, where Antp is normally not detected (Fig. 2F-H
211 and controls in Supplemental Fig. S3G-H'). This suggests that next to its auto-
212 repressing function, Antp is also capable of activating its own transcription (Fig. 2I).

213 To exclude that the auto-activation and repression of Antp are artifacts of
214 overexpression, we used FCS to measure the concentration of Antp triggered by
215 different Gal4-drivers (Supplemental Fig. S4A-E). We observed indistinguishable
216 DNA-binding behavior by FCS, not only across the whole concentration range
217 examined (Supplemental Fig. S4F), but also between endogenous and overexpressed
218 Antp (Supplemental Fig. S5A,B). Importantly, the auto-activating and auto-repressing
219 capacity of Antp was preserved even with the weak Gal4-driver *69B* (Supplemental
220 Fig. S4K,L) that triggered concentrations of Antp lower than its normal concentration
221 in the leg disc (473 nM versus 1110 nM), indicating that auto-activation and auto-
222 repression of Antp take place at endogenous protein concentrations.

223 We conclude that Antp is able to repress and activate its own transcription (Fig.
224 2E,I) and hypothesize that this auto-regulatory circuit sets the "correct" concentration
225 of Antp protein in imaginal discs.

226

227 A temporal switch controls the transition of *Antp* from a state of auto-activation to a 228 state of auto-repression

229 To further investigate the mechanism by which the Antp auto-regulatory circuit
230 sets the precise Antp expression levels, we next asked whether the seemingly
231 opposing auto-regulatory activities of Antp are separated in time during development.
232 To that end, we induced gain-of-function clones of full-length untagged *Antp* either at
233 26 h (first larval instar – henceforth referred to as "early" stage) or at 60 h (late second
234 larval instar – henceforth referred to as "late" stage) of development and analyzed the
235 clones in late third instar wing imaginal discs (Fig. 3). We chose these time points
236 based on Antp expression being widespread during first instar disc development and
237 therefore possibly amenable to auto-activation before becoming confined to the
238 proximal disc regions, whereas in the late second instar it is restricted to proximal only
239 regions (Emerald and Cohen, 2004). As a pre-requisite for this analysis, we
240 established that the Antp-eGFP homozygous viable MiMIC allele recapitulates the
241 endogenous Antp pattern in the embryo and all thoracic imaginal discs and therefore

242 can be used to monitor endogenous Antp protein (Supplemental Fig. S6). Clonal
243 induction of full-length untagged *Antp* in early development triggered strong auto-
244 activation of Antp-eGFP (Fig. 3A,B,B' and controls in Supplemental Fig. S7A-C'). As
245 before, we confirmed that early auto-activation of Antp is transcriptional and similar for
246 both full-length and SynthAntp proteins (Supplemental Fig. S7D-E' and controls in F-
247 G'). Early auto-activation was further supported by a loss-of-function experiment,
248 where *RNAi*-mediated early knockdown of Antp resulted in downregulation of the Antp
249 reporter (Fig. 3C,C' and controls in Supplemental Fig. S7H,H'). The loss and gain-of-
250 function analysis together suggest that during early disc development Antp is required
251 for sustaining its own expression.

252 In contrast, clonal induction during the late second instar stage (Fig. 3F)
253 repressed Antp-eGFP (Fig. H,H') and, reciprocally, the clonal knockdown by *RNAi*
254 triggered auto-activation of Antp transcription (Fig. 3I,I'). Hence, in contrast to early
255 development, Antp represses its own expression in third instar discs.

256 While the gain-of-function experiments show that Antp is sufficient to execute
257 auto-regulation, loss-of-function analysis indicates that it is also necessary for both
258 repression and activation at the transcriptional level.

259 Together, these results revealed the existence of a switch in Antp auto-
260 regulatory capacity on its own transcription during development. Starting from a
261 preferentially auto-activating state early in development (Fig. 3D), Antp changes to an
262 auto-inhibitory mode at later developmental stages (Fig. 3J).

263

264 During development Antp switches from a low-concentration/high-variability to a high- 265 concentration/low-variability state

266 If the *Antp* auto-repressive state limits the variability of Antp protein
267 concentration among neighboring cells late in development, we expected that the
268 variability would be higher during earlier stages, when auto-repression does not
269 operate. We, therefore, used FCS to characterize the endogenous expression levels
270 and cell-to-cell variability of Antp concentration in nuclei of second instar wing and leg
271 discs. We observed significantly lower average concentrations of Antp protein in
272 second versus third instar wing and leg discs and the inverse was true for
273 concentration variability (Fig. 3E and Supplemental Fig. S8A,A',C), indicating that the
274 developmental increase in concentration is accompanied by suppression of
275 concentration variability. In addition, FCS revealed a notable change in Antp
276 characteristic decay times (signifying molecular diffusion, limited by chromatin-
277 binding) at early versus late stages (Supplemental Fig. S8B). This behavior indicates
278 that endogenous Antp is initially moving fast in the nucleus, as it undergoes
279 considerably fewer interactions with chromatin compared to later stages where its
280 interactions with chromatin are more frequent and longer lasting.

281 Taken together, our measurements show that *Antp* is expressed at relatively
282 low and highly variable levels in early developing discs, when genetic evidence
283 indicates auto-activation capacity on its own transcription. Later in development, when
284 Antp has reached a state of higher average concentrations, auto-repression kicks in,
285 resulting in considerably lower variability among neighboring cells.

286

287 Dynamic control of Antp auto-regulation by different Antp isoforms

288 The changing binding behavior of Antp on chromatin from second to third instar
289 discs and the developmental transition from an auto-activating to an auto-repressing
290 state suggested a causal relationship between the two phenomena. We, therefore,
291 sought to identify molecular mechanisms that could link the observed changes in Antp

292 chromatin-binding to Antp auto-activation and repression. It is well established that the
293 Antp mRNA contains an alternative splice site in exon 7 immediately upstream of the
294 homeobox-containing exon 8, and generates Antp isoforms differing in as little as 4
295 amino acids in the linker between the YPWM motif (a cofactor-interacting motif) and
296 the homeodomain (Fig. 4A) (Stroeher et al., 1988). Our previous observation that long
297 linker isoforms favor transcriptional activation of Antp target genes, whereas short
298 linker isoforms favor repression of Antp targets (Papadopoulos et al., 2011), prompted
299 us to examine whether the linker length is also responsible for differences in auto-
300 regulation.

301 Ectopic expression of SynthAntp-eGFP peptides featuring a long linker
302 displayed significantly weaker repression capacity on endogenous Antp, as compared
303 to their short linker counterparts (Fig. 4B,B',F,F' and quantified in D,H, see also
304 Materials and Methods). We confirmed that, also in this case, the repression was at
305 the transcriptional level (Supplemental Fig. S9I-J'). Inversely, long linker Antp isoforms
306 exhibited stronger activation of Antp reporter, as compared to short linker isoforms
307 (Fig. 4C,C',G,G' and quantified in D,H; see also Materials and Methods). We,
308 additionally, validated that short linker isoforms encoded by full-length or SynthAntp
309 cDNAs behaved as weaker auto-activating and stronger auto-repressing Antp species
310 in all our previous experiments using the endogenous Antp protein and the P1 reporter
311 (Supplemental Fig. S9A-H'). We conclude that, also in the case of Antp auto-
312 regulation, short linker isoforms function as more potent repressors, whereas long
313 linker ones operate as more potent activators.

314 Since the Antp P1 promoter undergoes a switch from preferential auto-activation
315 to auto-repression, and short and long linker Antp isoforms function as preferential
316 auto-repressors and auto-activators, respectively, it appeared possible that the switch
317 in Antp regulation is executed at the level of transcript variant abundance of these
318 isoforms. Therefore, we next quantified the relative abundance of long and short linker
319 transcript variants in the embryo, second and third instar discs (Fig. 4D,H). The data
320 showed that the abundance of the long linker variant decreased, whereas the
321 abundance of the short linker variant increased over time in development, in line with
322 previous observations (Stroeher et al., 1988). Thus, as hypothesized, this finding
323 suggested that relative transcript variant abundance may underlie the switch between
324 auto-activation and auto-repression (without excluding additional mechanisms, such
325 as changes in the chromatin modifications between early and later disc development,
326 or the participation of different cofactors).

327 Relative changes in Antp transcript variant abundance (Fig. 4D,H), differential
328 efficiency of their encoded isoforms to repress or activate the *Antp* gene (Fig. 4B-D,F-
329 H), the developmental switch of *Antp* from auto-activation to repression (Fig. 3) and
330 the different mobility of Antp between second and third instar imaginal discs (Fig. 3E)
331 all pointed towards the hypothesis that the two isoforms have different modes of
332 interaction with chromatin. To investigate this, we expressed the two isoforms from the
333 *69B* enhancer in third instar wing and antennal discs. This results in Antp
334 concentrations close to (if not below) endogenous levels (Supplemental Fig. S4A-J).
335 FCS measurements revealed that the short linker isoform displayed longer
336 characteristic decay times and a higher fraction of DNA-bound molecules, suggesting
337 stronger and more pronounced binding to chromatin than its long linker counterpart
338 (Fig. 4D,H and Supplemental Fig. S10A,B). With chromatin (and therefore Antp
339 binding sites configuration), as well as the presence of cofactor proteins, being
340 identical between the two instances (short and long linker isoforms examined in third
341 instar wing and antennal imaginal discs of the same age), we were able to directly

342 compare the apparent equilibrium dissociation constants for the two isoforms
343 (Supplement 3). We found that the affinity of binding to chromatin (K_d^{-1}) of the
344 repressing short linker isoform is at least 2.3 times higher compared to the activating
345 long linker isoform ($\frac{K_{d, Antp}^{long linker isof.}}{K_{d, Antp}^{short linker isof.}} > 2.3$) (Fig. 4D,H and Supplemental Fig. S10C-D').

346 To corroborate these findings, we also performed gel-shift experiments to test how
347 full-length recombinant Antp isoforms, bearing a short and a long linker, bind
348 previously characterized Antp binding sites. We found that equal amounts of Antp long
349 linker isoform binds *Antp* binding sites weaker than its short linker counterpart
350 (Supplemental Fig. S11). Collectively, these experiments support the notion that
351 differences in Antp regulation during disc development can be largely attributed to
352 differences in the affinity of the investigated Antp isoforms.

353 Taken together, the switch of Antp from an auto-activating to an auto-repressing
354 state and the alteration of its DNA-binding behavior during disc development can be
355 largely explained by a temporal developmental regulation of the relative
356 concentrations of preferentially auto-activating and auto-repressing Antp protein
357 isoforms, which themselves display distinct properties in their modes of interaction
358 with chromatin (Fig. 4E,I).

359

360 Robustness of Antp auto-regulation

361 The mechanism of developmental Antp auto-regulation offered a possible
362 explanation for the observed increase in Antp concentration from second to third instar
363 discs, as well as the suppression of variability. What remained an open question is the
364 functional significance of suppression of Antp variability in development. To test this,
365 we require to manipulate variability, yet this is currently not possible to achieve at the
366 endogenous locus. However, since average concentration and variability are
367 interdependent, we used an ectopic expression system to progressively dampen Antp
368 variability by manipulating its concentration. To this end, we expressed SynthAntp
369 ectopically in the antennal disc, devoid of endogenous Antp expression, and monitored
370 the extent (strength) of homeotic transformations induced by different Gal4 drivers
371 corresponding to different SynthAntp concentrations (as measured by FCS previously
372 in Supplemental Fig. S4A-D). In this experiment, expression of SynthAntp is controlled
373 by the Gal4 driver, independently of the Antp locus, therefore the phenotypic output
374 does not depend on Antp auto-regulation. We observed that partial transformations of
375 antennae to tarsi could be obtained with drivers expressing Antp at close to
376 endogenous concentration (*ptc*-Gal4, *Dll*-Gal4 (MD713) and *69B*-Gal4 drivers, Fig.
377 5B-D and Supplemental Fig. S4B-D). Therefore, Antp can repress the antennal and
378 launch the leg developmental program in the antennal disc at endogenous
379 concentrations, although not robustly across the tissue (Supplement 5). As expected,
380 the three weak transformation phenotypes, elicited by *ptc*-, *Dll* (MD713)- and *69B*-Gal4
381 (Fig. 5B-D) were accompanied by high variability of SynthAntp concentration in
382 developing discs (Fig. 5E,F). In contrast, strong expression of SynthAntp from the *Dll*-
383 Gal4 (MD23) enhancer resulted in robust homeotic transformation to a complete
384 tarsus (Fig. 5A), accompanied by low cell to cell variability (Fig. 5F). This condition
385 resembled most closely the endogenous Antp variability in the leg disc ($CV^2 = 0.103$).
386 Importantly, endogenous Antp and Antp overexpressed by any of the Gal4 drivers
387 showed indistinguishable chromatin-binding behavior by FCS (Supplemental Fig. S4F
388 and Supplemental Fig. S5A,B). Therefore, robust Antp homeotic function can be
389 achieved at concentrations that are accompanied by low variability.

390 In order to further substantiate the qualitative model of Antp auto-regulation
391 suggested by our findings and examine its impact on protein variability, we developed
392 a simple mathematical model of stochastic *Antp* expression (Supplement 4). This
393 model tests whether positive and negative auto-regulation of Antp through distinct
394 isoforms is sufficient to explain the increase in protein concentration and decrease in
395 nucleus-to-nucleus variability from early to late stages. The model consists of a
396 dynamic promoter, which drives transcription of *Antp* followed by a splicing step,
397 yielding either the auto-repressing or the auto-activating isoform of Antp. Since the
398 repressing isoform has higher abundance at later stages, we assumed that splicing is
399 more likely to generate this isoform than the activating isoform. The initial imbalance
400 of Antp towards the activating isoform (Fig. 4D,H) is modeled through appropriate
401 initial concentrations of each isoform.

402 Since Antp copy numbers per nucleus are in the thousands at both early and
403 late stages, intrinsic noise of gene expression is likely to explain only a certain portion
404 of the overall variability in Antp concentrations (Elowitz et al., 2002; Taniguchi et al.,
405 2010). The remaining extrinsic variability is due to cell-to-cell differences in certain
406 factors affecting gene expression such as the ribosomal or ATP abundances. To check
407 whether extrinsic variability significantly affects Antp expression, we expressed
408 nuclear mRFP1 constitutively, alongside with endogenous Antp-eGFP, and measured
409 their abundances (Supplemental Fig. S12). With extrinsic factors affecting both genes
410 similarly, we expected a correlation between the concentration of nuclear mRFP1 and
411 Antp-eGFP. Our data showed a statistically significant correlation between mRFP1
412 and Antp (Supplemental Fig. S11C, $r = 0.524$ and $p = 9.77 \cdot 10^{-5}$). Correspondingly,
413 we accounted for extrinsic variability also in our model by allowing gene expression
414 rates to randomly vary between cells (Zechner et al., 2012).

415 The promoter itself is modeled as a Markov chain with three distinct
416 transcriptional states. In the absence of Antp, the promoter is inactive and transcription
417 cannot take place (state “U” in Fig. 5G). It can switch into a highly expressing state “A”
418 at a rate that is assumed to be proportional to the concentration of the auto-activating
419 isoform (Antp-A, Fig. 5G). This resembles the positive auto-regulatory function of Antp.
420 Conversely, the promoter can be repressed by recruitment of the auto-repressing
421 isoform, state “R” in the model (Antp-R, Fig. 5G). Since the auto-repressing isoform of
422 Antp can also activate the promoter, albeit significantly weaker than the auto-activating
423 isoform, and vice versa, we allow the promoter to switch between states “A” and “R”.

424 In this promoter model, it remained unclear whether the two isoforms compete
425 for the same binding sites on the P1 promoter. In this case, an increase in
426 concentration of repressing Antp species enhances the probability to reach state “R”
427 only if the promoter is in state “U” (Fig. 5G). In the absence of competitive binding, the
428 rate of switching between “A” and “R” also depends on the concentration of repressing
429 isoforms of Antp (Fig. 5G, compared to Supplemental Fig. S13A). We analyzed both
430 model variants by forward simulation and found that both of them can explain the
431 switch-like increase in average Antp concentration between early and late stages (Fig.
432 5J, compared to Supplemental Fig. S13D) and the relative fraction of repressing and
433 activating isoforms (Fig. 5I, compared to Supplemental Fig. S13C). However, only the
434 non-competitive binding model (Fig. 5G) can explain the substantial reduction of total
435 Antp variability between early and late stages (Fig. 5J, Supplemental Fig. S13D).
436 Simulation trajectories of individual nuclei indicated an initial increase and a
437 subsequent stabilization of concentration, whereas in the competitive model, or in the
438 absence of the negative feedback, this is not achieved (Fig. 5H, compared to
439 Supplemental Fig. S13B,F). Additionally, we established that the negative feedback is

440 required for suppression of variability (Supplemental Fig. S13E,H), as otherwise no
441 suppression of variability is conferred (Supplemental Fig. S13H). Thus, the model
442 suggested that auto-repression is required and that isoforms do not compete for
443 binding to the P1 promoter.

444 To further validate this model, we analyzed how Antp variability scales with
445 average concentrations, compared to our experimental measurements. To generate
446 different average concentrations, we varied the gene expression rates over three
447 orders of magnitude. The model predicted a decrease in variability as a function of
448 total Antp concentration and an increase in the Fano factor. These findings are in good
449 agreement with the experimental data (compare Fig. 5K to K' and L to L').

450 We next analyzed the model behavior under different genetic perturbations.
451 Increase of Antp concentration by overexpressing SynthAntp transgenes (bearing
452 either a long or a short linker isoform) from the Antp P1 promoter (Antp P1-Gal4>UAS-
453 SynthAntp-eGFP long or short linker) resulted in 100% embryonic lethality, rendering
454 the analysis of concentration and variability in imaginal discs impossible. This
455 indicated that indiscriminate increase of the dosage of either Antp variant from early
456 embryonic development onwards cannot be tolerated or buffered by the auto-
457 regulatory circuit.

458 However, overexpression from a *Dll* enhancer [*Dll*-Gal4 (MD23)] in the leg discs
459 or in the notum (*MS243*-Gal4), which overlaps with the endogenous Antp expression
460 pattern only during first instar disc development (Emerald and Cohen, 2004), resulted
461 in normal adult leg and notum structures. Flies overexpressing either the SynthAntp
462 auto-activating or the auto-repressing isoform in distal appendages (Fig. 6A,B) or the
463 notum (Supplemental Fig. S14A) displayed the wild type morphology, indicative of
464 normal Antp function, regardless of which isoform (activating or repressing) was
465 overexpressed. We further measured by FCS the concentration and variability of the
466 total Antp protein (endogenous Antp-eGFP and overexpressed SynthAntp-eGFP) in
467 proximal regions of the leg disc at second and third instar stages (Fig. 6C,C'). We
468 found that the concentration remained high at both stages due to the overexpression
469 but variability was reduced to endogenous levels at late stages. Also, the reduced Antp
470 variability does not seem to depend on Antp concentration alone, because for high
471 concentrations at both early and late stages, variability is high only in the early stage
472 but reduced in the late stage. Together, the phenotypic analysis and FCS
473 measurements indicate that Antp auto-regulation is able to reduce variability, even at
474 high levels of expression of either isoform, ensuring proper leg development.

475 The experimental data were corroborated by the model, which predicted that
476 more than three-fold overexpression of either auto-activating or auto-repressing Antp
477 isoforms (Fig. 6E,H) will nevertheless equilibrate to normal expression levels at later
478 stages (Fig. 6D,F,G,I). Specifically, we have measured by FCS roughly 15400
479 molecules in the wild type leg disc, and the model is in good quantitative agreement
480 with this measurement upon overexpression of the activating or repressing isoform. In
481 addition, there is no negative effect on the noise suppressing property of the circuit
482 (Fig. 6F,I). Thus, both the model and experimental data indicate that transient high
483 levels of either isoform early during disc development can be tolerated and that the
484 concentration and cell-to-cell variability of the endogenous Antp protein is restored at
485 later stages.

486 In contrast, overexpression of an exogenous repressor, such as Sex combs
487 reduced (*Scr*), which can repress Antp at the transcriptional level, but can neither
488 activate it nor activate its own transcription (Supplemental Fig. S14E-J'), resulted in
489 abnormal leg (Fig. 6J) and notum (Supplemental Fig. S14B) development. These flies

490 died as pharate adults with malformed legs, in line with *Antp* being required for proper
491 leg development in all ventral thoracic discs (legs). FCS measurements in the
492 corresponding proximal leg disc cell nuclei of second and third instar leg discs
493 overexpressing mCherry-SynthScr revealed pronounced reduction in *Antp*
494 concentration and remarkable increase in variability (Fig. 6K). In agreement, the model
495 predicted a similar block of transcription and correspondingly severe effects on *Antp*
496 dynamics (Fig. 6L-N). In both the measurements and the model prediction, the high
497 increase in variability was triggered by the fact that a majority of the cells do not
498 manage to switch into the highly-expressing state since too few long-linker *Antp*
499 molecules are present to establish the positive auto-regulation. Since splicing favors
500 the short-linker isoforms at later stages, these cells never “recover” from Scr
501 repression after restriction of the *Antp* overexpression domain to proximal regions of
502 the leg disc (Fig. 6L).

503 Taken together, the minimal model of *Antp* auto-regulatory genetic circuit is
504 able to explain the experimentally observed differences in *Antp* concentration and cell-
505 to-cell variability at early and late developmental stages.

506

507 Discussion

508 In this work, we found that *Antp* auto-regulates its expression levels
509 development, starting from a preferentially auto-activating state early and transitioning
510 to a preferentially auto-repressing state later. The early state is characterized by lower
511 average *Antp* concentrations and high variability, whereas the opposite is true for the
512 later repressing state. Without excluding other mechanisms, such as chromatin
513 configuration, accessibility of Hox binding sites to *Antp*, the differential abundance of
514 cofactors among developmental stages, or different modes of interactions with
515 different *Antp* isoforms, we have shown that differential expression of *Antp* isoforms is
516 one contributing mechanism for the observed regulatory switch. These isoforms have
517 preferentially activating or repressing activities on the *Antp* promoter, bind chromatin
518 with different affinities and are themselves expressed in different relative amounts
519 during development. A loss-of-function analysis of the isoforms *in vivo* will be required
520 to provide a definitive answer on the relative contribution of the *Antp* isoform-mediated
521 auto-regulatory circuit towards observed suppression of variability. CRISPR/Cas9-
522 mediated genome manipulation, in principle, allows the generation of *Antp* loci that
523 express only one or the other isoform. However, it is not clear whether these flies can
524 reach the larval developmental stages, given the *Antp* embryonic functions and, in
525 fact, strong biases towards only the activating or repressing isoform introduced by
526 *Antp*-Gal4-mediated expression of either *Antp* isoform resulted in embryonic lethality.
527 In the absence of such direct evidence, we turned to mathematical modelling and
528 derived, based on our experimental data, a simple kinetic model of *Antp* auto-
529 regulation that confirmed the plausibility of the proposed mechanism. In addition, the
530 model generated predictions that could be verified by introducing genetic
531 perturbations.

532 Negative auto-regulation has been identified as a frequently deployed
533 mechanism for the reduction of noise (cell-to-cell variability) and the increase of
534 regulatory robustness in various systems (Becskei and Serrano, 2000; Dublanche et
535 al., 2006; Gronlund et al., 2013; Nevozhay et al., 2009; Shimoga et al., 2013; Thattai
536 and van Oudenaarden, 2001). Auto-repression has been described for the Hox gene
537 *Ultrabithorax* (*Ubx*) in haltere specification and as a mechanism of controlling *Ubx*
538 levels against genetic variation (Crickmore et al., 2009; Garaulet et al., 2008), as well
539 as in *Ubx* promoter regulation in *Drosophila* S2 cells (Krasnow et al., 1989). In contrast,

540 an auto-activating mechanism is responsible for the maintenance of *Deformed*
541 expression in the embryo (Kuziora and McGinnis, 1988). These experiments suggest
542 similar mechanisms for establishing (auto-activation) or limiting (auto-repression) Hox
543 TF levels and variability in different developmental contexts.

544 Our data suggest that the developmental switch from auto-activation to auto-
545 repression is, at least in part, mediated by molecularly distinct Antp linker isoforms.
546 Differences in affinities of different Hox TF isoforms, based on their linker between the
547 YPWM motif and the homeodomain, have also been identified for the Hox TF Ubx.
548 Interestingly, its linker is also subject to alternative splicing at the RNA level (Reed et
549 al., 2010). In a similar way to Antp, the long linker Ubx isoform displays four to five fold
550 lower affinity of DNA binding, as compared to short linker isoforms, and the two
551 isoforms are not functionally interchangeable in *in vivo* assays. Finally, the Ubx linker
552 also affects the strength of its interaction with the Hox cofactor Extradenticle (Exd),
553 underscoring the functional importance of linker length in Hox TF function (Saadaoui
554 et al., 2011).

555 Mathematical modeling predicts that the Antp auto-regulatory circuit is robust
556 with respect to initial conditions and extrinsic noise by suppressing cell-to-cell
557 concentration variability even at high concentrations of any of the two Antp isoforms
558 (auto-repressing or auto-activating). This “buffering” capacity on cell-to-cell variability
559 is reflected in the ability of flies to tolerate more than 3-fold overexpression of Antp
560 without dramatic changes in endogenous Antp levels or generation of abnormal
561 phenotypes. Therefore, two different isoforms produced from the same gene with
562 opposing roles in transcriptional regulation and different auto-regulatory binding sites
563 on the gene’s promoter seem to suffice to create a robust gene expression circuit that
564 is able to “buffer” perturbations of the starting conditions.

565 So far, we have only been able to indiscriminately increase or decrease Antp
566 concentration at the tissue level and record the phenotypic outcome of these boundary
567 states. It will be interesting to test whether controlled perturbations of TF variability at
568 the tissue level that render TF concentration patterns less, or more, noisy among
569 neighboring cells, while maintaining similar mean protein concentrations, lead to
570 abnormal phenotypes. The technology to selectively manipulate expression variability
571 of specific TF in a developing tissue is yet to be established.

572

573 **Materials and Methods**

574

575 Fly stocks used

576 The Antp-eGFP MiMIC line has been a kind gift from Hugo J. Bellen
577 (Bloomington stock 59790). The *atonal* (VDRC ID 318959), *brinker* (VDRC ID
578 318246), *spalt major* (VDRC ID 318068), *yorkie* (VDRC ID 318237), *senseless* (VDRC
579 ID 318017) and *Sex combs reduced* (VDRC ID 318441) fosmid lines are available
580 from the Vienna Drosophila Resource Center (VDRC) and have been generated
581 recently in our laboratory (Sarov et al., 2016). The *fork head* (stock 43951), *grainy*
582 *head* (stock 42272), *Abdominal B* (stock 38625), *eyeless*, (stock 42271), *spineless*
583 (transcript variant A, stock 42289), and *grain* (stock 58483) tagged BACs were
584 generated by Rebecca Spokony and Kevin P. White and are available at the
585 Bloomington Stock Center. For the *scalloped* gene, a GFP-trap line was used
586 (Buszczak et al., 2007), a kind gift from Allan C. Spradling laboratory (line CA07575),
587 with which genome-wide chromatin immunoprecipitation experiments have been
588 performed (Slattery et al., 2013). For the *spineless* gene, Bloomington stock 42676,
589 which tags isoforms C and D of the Spineless protein has been also tried in

590 fluorescence imaging and FCS experiments, but did not yield detectable fluorescence
591 in the antennal disc, rendering it inappropriate to be used in our analysis. Therefore,
592 we resided to stock 42289, which tags the A isoform of the protein. For the *eyeless*
593 gene, the FlyFos015860(pRedFlp-Hgr)(ey13630::2XTY1-SGFP-V5-preTEV-BLRP-
594 3XFLAG)dFRT line (VDRC ID 318018) has been tried also in fluorescence imaging
595 and FCS experiments, but did not yield detectable fluorescence in the eye disc for it
596 to be used in our analysis. The *act5C-FRT-yellow-FRT-Gal4* (Ay-Gal4) line used for
597 clonal overexpression or *RNAi* knockdown has been described (Ito et al., 1997). The
598 UAS-*Antp* lines (synthetic and full-length), as well as UAS-SynthScr constructs have
599 been previously described (Papadopoulos et al., 2011; Papadopoulos et al., 2010).
600 The *Dll-Gal4* (MD23) line has been a kind gift of Ginés Morata (Calleja et al., 1996).
601 *69B-Gal4* and *ptc-Gal4* have been obtained from the Bloomington Stock Center. The
602 *Antp P1-lacZ* and *P2-lacZ* have been previously described (Engstrom et al., 1992; Zink
603 et al., 1991). The P1 reporter construct spans the region between 9.4 kb upstream of
604 the P1 promoter transcription initiation site and 7.8 kb downstream into the first intron,
605 including the first exon sequences and thus comprising 17.2 kb of *Antp* regulatory
606 sequences (pAPT 1.8). The line used has been an insertion of the pAPT 1.8 vector
607 bearing the P1 promoter regulatory sequences upstream of an *actin-lacZ* cytoplasmic
608 reporter and has been inserted in cytogenetic location 99F on the right chromosomal
609 arm of chromosome 3. The *Antp-RNAi* line has been from VDRC, line KK101774.
610 UAS-eGFP stock was a kind gift of Konrad Basler. We are indebted to Sebastian Dunst
611 for generating the *ubi-FRT-mCherry(stop)-FRT-Gal4(VK37)/CyO* line, which drives
612 clonal overexpression upon flippase excision, while simultaneously marking cells by
613 the loss of mCherry. For red-color labeling of clones the *act5C-FRT-CD2-FRT-Gal4*,
614 UAS-mRFP1(NLS)/TM3 stock 30558 from the Bloomington Stock Center has been
615 used. For marking the ectopic expression domain of untagged *Antp* proteins the UAS-
616 mRFP1(NLS)/TM3 stock 31417 from the Bloomington Stock Center has been used.
617 The *MS243-Gal4*; UAS-GFP/CyO line was a kind gift from the laboratory of Ernesto
618 Sánchez-Herrero.

619

620 Fly genotypes corresponding to fluorescence images

621 Supplemental Fig. S1A: FlyFos018487(pRedFlp-Hgr)(ato37785::2XTY1-SGFP-V5-
622 preTEV-BLRP-3XFLAG)dFRT

623 Supplemental Fig. S1B: FlyFos024884(pRedFlp-Hgr)(brk25146::2XTY1-SGFP-V5-
624 preTEV-BLRP-3XFLAG)dFRT

625 Supplemental Fig. S1C: FlyFos030836(pRedFlp-Hgr)(salm30926::2XTY1-SGFP-V5-
626 preTEV-BLRP-3XFLAG)dFRT

627 Supplemental Fig. S1: FlyFos029681(pRedFlp-Hgr)(yki19975::2XTY1-SGFP-V5-
628 preTEV-BLRP-3XFLAG)dFRT

629 Supplemental Fig. S1E: w^{1118} ; PBac(fkh-GFP.FPTB)VK00037/SM5

630 Supplemental Fig. S1F: *sd*-eGFP (FlyTrap, homozygous)

631 Supplemental Fig. S1G: w^{1118} ; PBac(grh-GFP.FPTB)VK00033

632 Supplemental Fig. S1H: FlyFos018974(pRedFlp-Hgr)(Scr19370::2XTY1-SGFP-V5-
633 preTEV-BLRP-3XFLAG)dFRT

634 Supplemental Fig. S1I: FlyFos015942(pRedFlp-Hgr)(sens31022::2XTY1-SGFP-V5-
635 preTEV-BLRP-3XFLAG)dFRT

636 Supplemental Fig. S1J,K: *Antp*-eGFP (MiMIC) homozygous (line MI02272, converted
637 to an artificial exon)

638 Supplemental Fig. S1L: w^{1118} ; PBac(Abd-B-EGFP.S)VK00037/SM5

639 Supplemental Fig. S1M: w^{1118} ; PBac(ey-GFP.FPTB)VK00033

640 Supplemental Fig. S1N: *w¹¹¹⁸*; PBac(ss-GFP.A.FPTB)VK00037
 641 Supplemental Fig. S1O,P: *w¹¹¹⁸*; PBac(grn-GFP.FPTB)VK00037
 642 Fig. 2B,B': *hs-flp/+*; *act5C-FRT-yellow-FRT-Gal4/+*; UAS-SynthAntp long linker-
 643 eGFP/+
 644 Fig. 2C,C': *hs-flp/+*; *act5C-FRT-yellow-FRT-Gal4*, UAS-eGFP/+; UAS-Antp long linker
 645 (full-length, untagged)/+
 646 Fig. 2G,G': *Dll-Gal4 (MD23)/+*; UAS-SynthAntp-eGFP/*Antp P1-lacZ*
 647 Supplemental Fig. S3A,A': *Antp P1-lacZ/TM3*
 648 Supplemental Fig. S3B,B': *Antp P2-lacZ/CyO*
 649 Supplemental Fig. S3C,C': wild type
 650 Supplemental Fig. S3D,D': *hs-flp*; *act5C-FRT-yellow-FRT-Gal4*, UAS-eGFP
 651 Supplemental Fig. S3E,E': *hs-flp/+*; *act5C-FRT-yellow-FRT-Gal4*, UAS-eGFP/+; *Antp*
 652 *P1-lacZ/+*
 653 Supplemental Fig. S3F,F': *hs-flp/+*; *act5C-FRT-yellow-FRT-Gal4*, UAS-eGFP/+; UAS-
 654 Antp long linker (full-length, untagged)/*Antp P1-lacZ*
 655 Supplemental Fig. S3G,G': *Dll-Gal4 (MD23)/+*; UAS-Antp long linker (full-length,
 656 untagged), UAS-mRFP1(NLS)/ *Antp P1-lacZ*
 657 Supplemental Fig. S3H,H': *Dll-Gal4 (MD23)/+*; UAS-mRFP1(NLS)/ *Antp P1-lacZ*
 658 Supplemental Fig. S4A: *Dll-Gal4 (MD23)/+*; UAS-SynthAntp long linker-eGFP/+
 659 Supplemental Fig. S4B: *ptc-Gal4/+*; UAS-SynthAntp long linker-eGFP/+
 660 Supplemental Fig. S4C: *Dll-Gal4 (MD713)/+*; UAS-SynthAntp long linker-eGFP/+
 661 Supplemental Fig. S4D,G,H,K: *69B-Gal4/UAS-SynthAntp long linker-eGFP*
 662 Supplemental Fig. S4I,J,L: *69B-Gal4/UAS- eGFP*
 663 Fig. 3B,B',G,G': *hs-flp/+*; *ubi-FRT-mChery-FRT-Gal4/+*; Antp-eGFP (MiMIC)/UAS-
 664 Antp long linker (full-length, untagged)
 665 Fig. 3C,C': *hs-flp/+*; UAS-Antp^{*RNAi*}/+; *Antp P1-lacZ/act5C-FRT-CD2-FRT-Gal4*, UAS-
 666 mRFP1(NLS)
 667 Fig. 3H,H': *hs-flp/+*; UAS-Antp^{*RNAi*}/*act5C-FRT-yellow-FRT-Gal4*, UAS-eGFP; *Antp P1-*
 668 *lacZ/+*
 669 Supplemental Fig. S6B: *Antp P1-lacZ/TM6B*
 670 Supplemental Fig. S7A,A': *hs-flp/+*; *ubi-FRT-mChery-FRT-Gal4/+*; Antp-eGFP
 671 (MiMIC)/UAS-Antp long linker (full-length, untagged)
 672 Supplemental Fig. S7B-C': *hs-flp/+*; *ubi-FRT-mChery-FRT-Gal4/+*; Antp-eGFP
 673 (MiMIC)/+
 674 Supplemental Fig. S7D,D': *hs-flp/+*; *act5C-FRT-yellow-FRT-Gal4*, UAS-eGFP/+; *Antp*
 675 *P1-lacZ/UAS-Antp long linker (full-length, untagged)*
 676 Supplemental Fig. SE,E': *hs-flp/+*; *act5C-FRT-yellow-FRT-Gal4/+*; UAS-SynthAntp
 677 long linker-eGFP/+
 678 Supplemental Fig. S7F,F': *hs-flp/+*; *act5C-FRT-yellow-FRT-Gal4*, UAS-eGFP/+
 679 Supplemental Fig. S7G,G': *hs-flp/+*; *act5C-FRT-yellow-FRT-Gal4*, UAS-eGFP/+; *Antp*
 680 *P1-lacZ/+*
 681 Supplemental Fig. S7H,H': *hs-flp/+*; UAS-Antp^{*RNAi*}/+; Antp-eGFP (MiMIC)/*act5C-FRT-*
 682 *CD2-FRT-Gal4*, UAS-mRFP1(NLS)
 683 Fig. 4B,B': *ptc-Gal4/+*; UAS-SynthAntp long linker-eGFP/+
 684 Fig. 4C,C': *Dll-Gal4 (MD23)/+*; UAS-SynthAntp long linker-eGFP/*Antp P1-lacZ*
 685 Fig. 4F,F': *ptc-Gal4/+*; UAS-SynthAntp long linker-eGFP/+
 686 Fig. 4G,G': *Dll-Gal4 (MD23)/+*; UAS-SynthAntp short linker-eGFP/*Antp P1-lacZ*
 687 Supplemental Fig. S9A,A': *hs-flp/+*; *act5C-FRT-yellow-FRT-Gal4/+*; UAS-SynthAntp
 688 short linker-eGFP/+

689 Supplemental Fig. S9B,B',G,G': *hs-flp/+; act5C-FRT-yellow-FRT-Gal4/+; UAS-*
690 *SynthAntp short linker-eGFP/Antp P1-lacZ*
691 Supplemental Fig. S9C,C',H,H': *hs-flp/+; act5C-FRT-yellow-FRT-Gal4/+; UAS-Antp*
692 *short linker (full-length, untagged)/Antp P1-lacZ*
693 Supplemental Fig. S9D,D': *hs-flp/+; Dll-Gal4 (MD23)/+; UAS-Antp short linker (full-*
694 *length, untagged), UAS-mRFP1(NLS)/Antp P1-lacZ*
695 Supplemental Fig. S9E-F': *hs-flp/+; ubi-FRT-mChery-FRT-Gal4/+; Antp-eGFP*
696 *(MiMIC)/UAS-Antp short linker (full-length, untagged)*
697 Supplemental Fig. S9I,I': *ptc-Gal4/+; UAS-SynthAntp long linker-eGFP/Antp P1-lacZ*
698 Supplemental Fig. S9J,J': *ptc-Gal4/+; UAS-SynthAntp short linker-eGFP/Antp P1-lacZ*
699 Fig. 5A: *Dll-Gal4 (MD23)/+; UAS-SynthAntp long linker-eGFP/+*
700 Fig. 5B: *ptc-Gal4/+; UAS-SynthAntp long linker-eGFP/+*
701 Fig. 5C: *Dll-Gal4 (MD713)/+; UAS-SynthAntp long linker-eGFP/+*
702 Fig. 5D: *69B-Gal4/UAS-SynthAntp long linker-eGFP*
703 Supplemental Fig. S12A-B': *ubi-mRFP1(NLS)/+ or y; Antp-eGFP (MiMIC)/+*
704 Supplemental Fig. S13B,C: *Dll-Gal4 (MD23)/+; UAS-mCitrine-SynthScr/+*
705 Fig. 6A,B: *Dll-Gal4 (MD23)/+; UAS-SynthAntp long linker-eGFP/+ or Dll-Gal4*
706 *(MD23)/+; UAS-SynthAntp short linker-eGFP/+*
707 Fig. 6J: *Dll-Gal4 (MD23)/+; UAS-mCitrine-SynthScr/+*
708 Supplemental Fig. S14A,D,D': *MS243-Gal4/+; UAS-SynthAntp long linker-eGFP/Dr or*
709 *MS243-Gal4/+; UAS-SynthAntp short linker-eGFP/Dr*
710 Supplemental Fig. S14B,F,F': *MS243-Gal4/+; UAS-mCitrine-SynthScr/+*
711 Supplemental Fig. S14C,I,I': *Dll-Gal4 (MD23)/+; UAS-mCitrine-SynthScr/+*
712 Supplemental Fig. S14E,E': *ptc-Gal4/+; UAS-SynthAntp long linker-eGFP/+*
713 Supplemental Fig. S14F,F': *MS243-Gal4/+; UAS- mCitrine-SynthScr/+*
714 Supplemental Fig. S14G,G': *ptc-Gal4/+; UAS- mCitrine-SynthScr/Antp P1-lacZ*
715 Supplemental Fig. S14H,H': *Dll-Gal4 (MD23)/+; UAS-mCitrine-SynthScr/Antp P1-lacZ*
716 Supplemental Fig. S14J,J': *MS243-Gal4/+; UAS-eGFP/+*

717

718 Preparation of second and third instar imaginal discs for FCS measurements

719 For FCS measurements, imaginal discs (eye-antennal, wing, leg, humeral and
720 genital) and salivary glands were dissected from third instar wandering larvae, or wing
721 and leg discs from second instar larvae, in Grace's insect tissue culture medium
722 (ThermoFisher Scientific, 11595030) and transferred to 8-well chambered coverglass
723 (Nunc® Lab-Tek™, 155411) containing PBS just prior to imaging or FCS
724 measurements. Floating imaginal discs or salivary glands were sunk to the bottom of
725 the well using forceps.

726

727 Immunostainings in larval imaginal discs

728 Larval imaginal discs were stained according to (Papadopoulos et al., 2010).
729 Stainings for the endogenous Antp protein have been performed using a mouse anti-
730 Antp antibody (Developmental Studies Hybridoma Bank, University of Iowa, anti-Antp
731 4C3) in a dilution of 1:250 for embryos and 1:500 for imaginal discs. eGFP, or eGFP-
732 tagged proteins have been stained using mouse or rabbit anti-GFP antibodies from
733 ThermoFisher Scientific in a dilution of 1:500 in imaginal discs and 1:250 in embryos.
734 mRFP1 was stained using a Chromotek rat anti-RFP antibody. For *Antp P1* promoter
735 stainings in imaginal discs we used the mouse anti- β -galactosidase 40-1a antibody
736 from Developmental Studies Hybridoma Bank, University of Iowa in a dilution of 1:50.
737 The rabbit anti-Scr antibody was used in a dilution of 1:300 (LeMotte et al., 1989).
738 Confocal images of antibody stainings represent predominately Z-projections and Zeiss

739 LSM510, Zeiss LSM700 or Zeiss LSM880 Airyscan confocal laser scanning
740 microscopy systems with an inverted stand Axio Observer microscope were used for
741 imaging. Image processing and quantifications have been performed in Fiji (Schindelin
742 et al., 2012). For optimal spectral separation, secondary antibodies coupled to
743 Alexa405, Alexa488, Alexa594 and Cy5 (ThermoFischer Scientific) were used.

744

745 Colocalization of wild type and eGFP-tagged MiMIC Antp alleles in imaginal discs

746 To examine whether the pattern of the MiMIC Antp-eGFP fusion protein
747 recapitulates the Antp wild type expression pattern in both embryo and larval imaginal
748 discs, we performed immunostainings of heterozygous Antp-eGFP and wild type flies
749 to visualize the embryonic (stage 13) and larval expression of *Antp* and eGFP. In this
750 experiment, we 1) visualized the overlap between eGFP and *Antp* (the eGFP pattern
751 reflects the protein encoded by the MiMIC allele, whereas the *Antp* pattern reflects the
752 sum of protein produced by the MiMIC allele and the allele of the balancer
753 chromosome) and 2) compared the eGFP expression pattern to the Antp expression
754 pattern in wild type discs and embryos.

755

756 Induction of early and late overexpression and RNAi-knockdown clones in imaginal 757 discs

758 Genetic crosses with approximately 100 virgin female and 100 male flies were
759 set up in bottles and the flies were allowed to mate for 2 days. Then, they were
760 transferred to new bottles and embryos were collected for 6 hours at 25°C. Flies were
761 then transferred to fresh bottles and kept until the next collection at 18°C. To assess
762 Antp auto-activation, the collected eggs were allowed to grow at 25°C for 26 h from
763 the midpoint of collection, when they were subjected to heat-shock by submersion to
764 a water-bath of 38°C for 30 min and then placed back at 25°C until they reached the
765 stage of third instar wandering larvae, when they were collected for dissection, fixation
766 and staining with antibodies. To assess Antp auto-repression, the same procedure
767 was followed, except that the heat-shock was performed at 60 h of development after
768 the midpoint of embryo collection. Whenever necessary, larval genotypes were
769 selected under a dissection stereomicroscope with green and red fluorescence filters
770 on the basis of *deformed (dfd)*-YFP bearing balancer chromosomes (Le et al., 2006)
771 and visual inspection of fluorescence in imaginal discs.

772

773 Measurement of Antp transcript variant abundance

774 The linker between the Antp YPWM motif and the homeodomain contains the
775 sequence RSQFGKCQE. Short linker isoforms encode the sequence RSQFE,
776 whereas long linker isoforms are generated by alternative splicing of a 12 base pair
777 sequence encoding the four amino acid sequence GKCQ into the mRNA. We initially
778 designed primer pairs for RT-qPCR experiments to distinguish between the short and
779 long linker mRNA variants. For the short linker variant, we used nucleotide sequences
780 corresponding to RSQFERKR (with RKR being the first 3 amino acids of the
781 homeodomain). For detection of the long linker variant we designed primers either
782 corresponding to the RSQFGKCQ sequence, or GKCQERKR. We observed in control
783 PCRs (using plasmid DNA harboring either a long or a short linker cDNA) that primers
784 designed for the short linker variant still amplified the long linker one. Moreover, with
785 linker sequences differing in only four amino acids, encoded by 12 base pairs, primer
786 pairs flanking the linker could also not be used, since, due to very similar sizes, both
787 variants would be amplified in RT-qPCR experiments with almost equal efficiencies.
788 Therefore, we used primer pairs flanking the linker region to indiscriminately amplify

789 short and long linker variants, using non-saturating PCR (18 cycles) on total cDNA
790 generated from total RNA. We then resolved and assessed the relative amounts of
791 long and short linker amplicons in a second step using Fragment Analyzer (Advanced
792 Analytical). RNA was extracted from stage 13 embryos, second instar larvae at 60 h
793 of development, and leg or wing discs from third instar wandering larvae using the
794 Trizol[®] reagent (ThermoFischer Scientific), following the manufacturer's instructions.
795 Total RNA amounts were measured by NanoDrop and equal amounts were used to
796 synthesize cDNA using High-Capacity RNA-to-cDNA[™] Kit (ThermoFischer Scientific),
797 following the manufacturer's instructions. Total cDNA yields were measured by
798 NanoDrop and equal amounts were used in PCR, using in-house produced Taq
799 polymerase. 10 ng of plasmid DNA, bearing either a long or a short transcript cDNA
800 were used as a control. PCR product abundance was analyzed both by agarose gel
801 electrophoresis and using Fragment Analyzer (Advanced Analytical).

802 The quantification of the transcript variant concentration (Fig. 4 D and H) has
803 been made considering 100% (value equal to 1 on the y axis) as the sum of long and
804 short isoforms at each developmental stage, whereas the quantification of the relative
805 activation and repression efficiency has been performed considering the short linker
806 variant as having 100% repression and the long linker variant as having 100%
807 activation (values equal to 1 on the y-axis) efficiency.

808

809 Quantification of the relative repressing and activating efficiencies of different Antp 810 isoforms

811 Quantification of the relative efficiency of Antp activating and repressing
812 isoforms (Fig. 4D,H) were performed in Fiji (Schindelin et al., 2012) by outlining the
813 total region of repression or activation of Antp protein or P1 reporter staining and
814 quantification of the relative fluorescence intensity of the selected regions. From the
815 calculated values, we have subtracted the values obtaining by outlining and calculating
816 Antp protein or reporter beta-galactosidase staining background in the region of
817 expression of an eGFP transgene alone (negative control). 5-7 imaginal disc images
818 per investigated genotype were used for analysis. For the repression assay the
819 obtained values have been normalized over the intensity of Antp protein calculated in
820 the region of overlap between an eGFP expressing transgene and Antp (negative
821 control). In both cases (repression and activation), the highest efficiency per transcript
822 variant (for repression, the short linker isoform; for activation the long linker isoform)
823 have been set to 100%.

824

825 Fluorescence Microscopy Imaging of live imaginal discs and FCS

826 Fluorescence imaging and FCS measurements were performed on two
827 uniquely modified confocal laser scanning microscopy systems, both comprised of the
828 ConfoCor3 system (Carl Zeiss, Jena, Germany) and consisting of either an inverted
829 microscope for transmitted light and epifluorescence (Axiovert 200 M); a VIS-laser
830 module comprising the Ar/ArKr (458, 477, 488 and 514 nm), HeNe 543 nm and HeNe
831 633 nm lasers and the scanning module LSM510 META; or a Zeiss LSM780 inverted
832 setup, comprising Diode 405 nm, Ar multiline 458, 488 and 514 nm, DPSS 561 nm
833 and HeNe 633 nm lasers. Both instruments were modified to enable detection using
834 silicon Avalanche Photo Detectors (SPCM-AQR-1X; PerkinElmer, USA) for imaging
835 and FCS. Images were recorded at a 512X512 pixel resolution. C-Apochromat 40x/1.2
836 W UV-VIS-IR objectives were used throughout. Fluorescence intensity fluctuations
837 were recorded in arrays of 10 consecutive measurements, each measurement lasting
838 10 s. Averaged curves were analyzed using the software for online data analysis or

839 exported and fitted offline using the OriginPro 8 data analysis software (OriginLab
840 Corporation, Northampton, MA). In either case, the nonlinear least square fitting of the
841 autocorrelation curve was performed using the Levenberg–Marquardt algorithm.
842 Quality of the fitting was evaluated by visual inspection and by residuals analysis.
843 Control FCS measurements to assess the detection volume were routinely performed
844 prior to data acquisition, using dilute solutions of known concentration of Rhodamine
845 6G and Alexa488 dyes. The variability between independent measurements reflects
846 variability between cells, rather than imprecision of FCS measurements. For more
847 details on Fluorescence Microscopy Imaging and FCS, refer to Supplement 1.

848 In Figure 1A-H the workflow of FCS measurements is schematically
849 represented. Live imaging of imaginal discs, expressing endogenously-tagged TFs,
850 visualized by fluorescence microscopy and neighboring cells, expressing TFs at
851 different levels, selected for FCS measurements (Fig. 1A-B). FCS measurements are
852 performed by placing the focal point of the laser light into the nucleus (Fig 1C-D) and
853 recording fluorescence intensity fluctuations (Fig. 1E), generated by the increase or
854 decrease of the fluorescence intensity, caused by the arrival or departure of fast- and
855 slowly-diffusing TF molecules into or out of the confocal detection volume (Fig. 1D).
856 The recorded fluctuations are subjected to temporal autocorrelation analysis, which
857 generates temporal autocorrelation curves (henceforth referred to as FCS curves),
858 which by fitting with an appropriate model (Supplement 1), yield information about the
859 absolute concentration of fluorescent molecules (F) and, after normalization to the
860 same amplitude, their corresponding diffusion times, as well as the fraction of fast-
861 and slowly-diffusing TF molecules (Fig. 1G). The concentration of molecules is
862 inversely proportional to the y-axis amplitude at the origin of the FCS curve (Fig. 1F).
863 Processes that slow down the diffusion of TF molecules, such as binding to very large
864 molecules (e.g. chromosomal DNA), are visible by a shift of the FCS curves to longer
865 characteristic times (Fig. 1G). Measurements in a collection of neighboring cell nuclei
866 also allow the calculation of protein concentration variability at the live tissue level (Fig.
867 1H).

868

869 Sample size, biological and technical replicates

870 For the measurement of TF molecular numbers and variability (Fig. 1 and
871 Supplemental Fig. S1), 7-10 larvae of each fly strain were dissected, yielding at least
872 15 imaginal discs, which were used in FCS analysis. For the Fkh TF, 7 pairs of salivary
873 glands were analyzed and for AbdB, 12 genital discs were dissected from 12 larvae.
874 More than 50 FCS measurements were performed in patches of neighboring cells of
875 these dissected discs, in the regions of expression indicated in Supplemental Fig. S1
876 by arrows. Imaginal discs from the same fly strain (expressing a given endogenously-
877 tagged TF) were analyzed on at least 3 independent instances (FCS sessions), taking
878 place on different days (biological replicates) and for Antp, which was further analyzed
879 in this study, more than 20 independent FCS sessions were used. As routinely done
880 with FCS measurements in live cells, these measurements were evaluated during
881 acquisition and subsequent analysis and, based on their quality (high counts per
882 molecule and second, low photobleaching), were included in the calculation of
883 concentration and variability. In Supplemental Fig. S1Q, n denotes the number of FCS
884 measurements included in the calculations.

885 For experiments involving immunostainings in imaginal discs to investigate the
886 auto-regulatory behavior of Antp (Figs. 2-5 and supplements thereof, except for the
887 temporally-resolved auto-activating and repressing study of Antp in Fig. 3, as
888 discussed above), 14-20 male and female flies were mated in bottles and 10 larvae

889 were selected by means of fluorescent balancers and processed downstream. Up to
 890 20 imaginal discs were visualized by fluorescence microscopy and high resolution Z-
 891 stacks were acquired for 3-5 representative discs or disc regions of interest per
 892 experiment. All experiments were performed in triplicate, except for the temporal
 893 analysis of Antp auto-regulatory behavior in Fig. 3 (and Supplemental Figs. thereof),
 894 which was performed 6 times and the quantification of repression efficiency of short
 895 and long linker Antp isoforms in Fig. 4 (and Supplemental Figs. thereof), which was
 896 performed 5 times.

897 For the quantification of transcript variant abundance in Fig. 4D,H, RNA and
 898 thus cDNA was prepared from each stage 3 independent times (biological replicates)
 899 and the transcript abundance per RNA/cDNA sample was also analyzed 3 times.
 900 For the experiments involving perturbations in Antp expression whereby the proper
 901 development of the leg and the notum have been assessed in Fig. 5, more than 100
 902 adult flies have been analyzed and this experiment has been performed more than 10
 903 times independently.

904 Statistical significance

905 Fig. 2D: Statistical significance was determined using a two-tailed Student's T-test (***,
 906 $p < 0.001$ and *, $p < 0.05$, namely $p_{Antp\ protein}^{repression\ clone\ vs\ surrounding} = 1.36 \cdot 10^{-15}$ and
 907 $p_{Antp\ reporter}^{repression\ clone\ vs\ surrounding} = 3.17 \cdot 10^{-16}$).

908 Fig. 2I: Statistical significance was determined using a two-tailed Student's T-test (***,
 909 $p < 0.001$ and *, $p < 0.05$, namely $p_{Antp\ reporter}^{Dll\ expression\ domain\ vs\ surrounding} = 1.55 \cdot 10^{-17}$).

910 Fig. 3E: Statistical significance was determined using a two-tailed Student's T-test (***,
 911 $p < 0.001$ and *, $p < 0.05$, namely $p_{Antp\ protein}^{early\ activation\ clone\ vs\ surrounding} = 6.23 \cdot 10^{-13}$ and
 912 $p_{Antp\ reporter}^{early\ knockdown\ (RNAi)\ clone\ vs\ surrounding} = 2.98 \cdot 10^{-9}$).

913 Fig. 3F: Statistical significance was determined using a two-tailed Student's T-test (***,
 914 $p < 0.001$ and *, $p < 0.05$, namely $p_{concentration}^{2nd\ vs\ 3rd\ instar} = 2.04 \cdot 10^{-20}$, $p_{\tau D2}^{2nd\ vs\ 3rd\ instar} =$
 915 $7.2 \cdot 10^{-4}$ and $p_{variation}^{2nd\ vs\ 3rd\ instar} = 3.4 \cdot 10^{-2}$).

916 Fig. 3K: Statistical significance was determined using a two-tailed Student's T-test (***,
 917 $p < 0.001$ and *, $p < 0.05$, namely $p_{Antp\ protein}^{late\ repression\ clone\ vs\ surrounding} = 3.98 \cdot 10^{-17}$ and
 918 $p_{Antp\ reporter}^{late\ knockdown\ (RNAi)\ clone\ vs\ surrounding} = 1.16 \cdot 10^{-21}$).

919 Fig. 4D, H: Statistical significance was determined using a two-tailed Student's T-test
 920 between measurements performed with the long linker (auto-activating) isoform (Fig.
 921 4D) and the short linker (auto-repressing) isoform (Fig. 4H) (***, $p < 0.001$ and *, $p <$
 922 0.05 , namely $p_{concentration}^{embryo\ long\ vs\ short} = 3.16 \cdot 10^{-5}$, $p_{concentration}^{2nd\ instar\ long\ vs\ short} = 1.16 \cdot 10^{-4}$,
 923 $p_{concentration}^{3rd\ instar\ long\ vs\ short} = 2.85 \cdot 10^{-6}$, $p_{relative\ activation}^{long\ vs\ short} = 4.1 \cdot 10^{-3}$, $p_{relative\ repression}^{long\ vs\ short} =$
 924 $2.4 \cdot 10^{-4}$ and $p_{DNA-bound\ fraction\ (FCS)}^{long\ vs\ short} = 5.6 \cdot 10^{-10}$).

925 Fig. 6C-C': Statistical significance was determined using a two-tailed Student's T-test
 926 ($p_{leg\ disc,\ o/e\ activator}^{early\ vs\ late\ conc.} = 0.679$ and $p_{leg\ disc,\ o/e\ repressor}^{early\ vs\ late\ conc.} = 0.454$).

927 Fig. 6K: Statistical significance was determined using a two-tailed Student's T-test
 928 ($p_{leg\ disc,\ o/e\ exog.\ repr.}^{early\ vs\ late\ conc.} = 0.892$).

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942

943 **Competing interests**

944 No competing interests declared.

945

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952

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1184
1185 **Figure Legends**

1186 **Figure 1: Concentration, DNA-binding dynamics and cell-to-cell protein**
1187 **concentration variability of 14 Drosophila TFs.** (A-H) Workflow of the study of TFs
1188 by FCS (see Materials and Methods and Supplement 1). (I) Representative average
1189 FCS measurements of eight TFs. (J) FCS curves shown in (I), normalized to the same
1190 amplitude, $G_n(\tau) = 1$ at $\tau = 10 \mu\text{s}$. (K) Variability of the 14 TFs as a function of
1191 concentration. (L) Variability in concentration of endogenous Antp in the wing disc. (M)
1192 Variability of Antp concentration in clusters of neighboring cell nuclei as a function of
1193 its average concentrations. Error bars in (K) and (N) represent 1 standard deviation.

1194
1195 **Figure 2: Antp activates and represses its own transcription.** (A) Schematic
1196 representation of the wing disc region of highest Antp expression (green cells). Antp
1197 is highly expressed in the wing disc in the regions of the notum that correspond to the
1198 structure of the prescutum in the adult cuticle, as well as in the base of the wing blade,
1199 which gives rise to the mesopleura and pteropleura of the adult thoracic cuticle. The
1200 black rectangle indicates the region of clonal analysis in (B-C'). (B-B') Clonal
1201 overexpression of a *SynthAntp-eGFP* construct. Dashed line in (B) shows a clone in
1202 the Antp expression domain. (C-C') Transcriptional auto-repression of Antp using the
1203 *Antp P1-lacZ*. (D) Quantification of repression of Antp protein and reporter inside the
1204 repression clones, as compared to the surrounding tissue. (E) Schematic of Antp
1205 transcriptional auto-repression. Repression can be direct or indirect. (F) Wing disc
1206 region of ectopic Antp P1 reporter expression in (G-G'). (G-G') Ectopic induction of
1207 *Antp P1-lacZ* in distal compartments of the wing disc by expression of *SynthAntp-*
1208 *eGFP* using *Dll-Gal4* (MD23). (H) Quantification of auto-activation of Antp reporter
1209 within the *Dll-Gal4* expression domain, as compared to the surrounding tissue. (I)
1210 Schematic representation of Antp auto-activation. Scale bars denote $100 \mu\text{m}$.

1211
1212 **Figure 3: Antp switches from transcriptional auto-activation to auto-repression.**
1213 (A, F) Clone induction at 26 h (early) or 60 h (late) with analysis at third instar larval
1214 stage (~96-120 h of development). Black rectangles represent the corresponding
1215 regions of clonal analysis. (B-B') Early clonal induction of full-length, untagged *Antp*,
1216 (mCherry positive) reveals strong auto-activation of endogenous Antp-eGFP (dashed
1217 lines in (B)). The cyan line outlines the region of highest endogenous Antp expression.
1218 The whole *Antp* expression domain expresses *Antp-eGFP*, but overexpression clones
1219 (sub-regions marked by absence of mCherry staining) express *Antp-eGFP* much
1220 stronger (B'). (C-C') *Antp P1* transcription in *Antp RNAi* knockdown clones (early clonal
1221 induction, dashed line in (C), marked by nuclear mRFP1. (D) Updated Antp auto-
1222 activation model, showing strong auto-activation of Antp at early stages. (E)
1223 Concentration, DNA-binding and variability studied by FCS at second instar leg and
1224 wing discs (FCS analysis in Supplemental Fig. S8). Low concentration, low degree of
1225 DNA-binding and high variability are observed in second instar wing and leg discs, but
1226 the opposite is true for third instar discs. (G-G') Late-induced clones (dashed lines in

1227 (G), expressing full-length, untagged *Antp* (mCherry positive). Auto-repression of Antp
1228 (dashed lines in (G)) is monitored by the endogenous Antp-eGFP protein. The cyan
1229 lines in (G) outline the region of strong endogenous expression. (H-H') *Antp* P1
1230 transcription in late *Antp RNAi* knockdown clones (dashed line in (H), 60 h of
1231 development) within the Antp normal expression domain, marked by nuclear mRFP1.
1232 The cyan line in (H) outlines the region of strong endogenous expression of the P1
1233 reporter. Cytoplasmic eGFP marks the *Antp* knockdown clone (H'). (I) Updated Antp
1234 auto-repression model showing the pronounced auto-repressing capacity of Antp at
1235 late stages.

1236

1237 **Figure 4: Antp auto-activation and auto-repression relies on Antp isoforms with**
1238 **different binding affinities to chromatin.** (A) Schematic of the Antp mRNA,
1239 generated from the P1 promoter. Exons are represented by grey boxes. Magnified
1240 exons 4-7 (drawn to scale, omitting splicing points for simplicity) show the alternative
1241 splice site (3' of exon 7), resulting in isoforms featuring a short linker between the
1242 YPWM motif and the homeodomain (RSQF, grey box), or a long linker isoform
1243 (RSQFGKCQ, white box). (B-B') *SynthAntp-eGFP* bearing a long linker expressed by
1244 *ptc-Gal4* and endogenous Antp protein auto-repression were monitored at the
1245 proximal portion of the wing disc. A white dashed line outlines the region of auto-
1246 repression that was used for quantification (see Materials and Methods). (C-C') Similar
1247 to B-B', except that expression was induced by *Dll* (MD23)-Gal4 distally (yellow
1248 dashed line). (D) Abundance of long linker isoform (see Materials and Methods); auto-
1249 activation and auto-repression efficiencies (Materials and Methods); DNA-bound
1250 fractions, measured by FCS ([Supplemental Figure S10](#)); and relative affinity of binding
1251 to chromatin, calculated by FCS ([Supplemental Figure S10](#)) are presented for
1252 comparison with (H). (E) Updated model of *Antp* auto-regulation. The activating
1253 isoform binds with lower affinity to the P1 Antp promoter, but is produced in excess,
1254 relative to the repressing one, resulting in preferential activation of transcription. (F-
1255 G') Similar to (B-C') for the short linker isoform. (H) Similar to (D) for comparison. (I)
1256 Updated qualitative model representation of Antp repression as in (E), whereby at later
1257 stages excess of Antp auto-repressor accounts for negative feedback on
1258 transcriptional regulation of the P1 promoter, resulting in partial activation of
1259 transcription, hence expression is maintained.

1260

1261 **Figure 5: Concentrations resulting in low variability are required for Antp**
1262 **homeotic function.** (A-D) Transformations of the distal antenna into a tarsus in adult
1263 flies, caused by *SynthAntp-eGFP* overexpression in antennal discs ([Supplemental](#)
1264 [Figure S4A-D](#)). Ectopic tarsi range from complete (A) to milder transformations of the
1265 arista (B and C) or ectopic leg bristles in the third antennal segment in (C, D) (black
1266 arrows). (E-F) Measurements of SynthAntp concentration and cell-to-cell variability of
1267 antennal discs ([Supplemental Figure S4A-D](#)) in the corresponding antennal discs (A-
1268 D). The three Gal4 drivers (blue font) result in partial transformations, despite being
1269 expressed at similar levels as the wild type Antp protein in the leg disc. However, their
1270 variability is higher than the endogenous variability ($CV^2 = 0.1$). In contrast, *Dll*-Gal4
1271 (MD23), results in much more robust homeotic transformations (A), accompanied by
1272 the lowest variability and closest to the wild type condition. (G-J) A dynamic promoter,
1273 which drives transcription of *Antp* followed by a splicing step, leads to either the
1274 repressing ("R" in (G)) or activating ("A" in (G)) isoform of Antp. In the absence of Antp,
1275 the promoter is inactive and transcription cannot take place ("U" in (G)). This promoter
1276 configuration leads to suppression of variability and increase in Antp concentration (J).

1277 Trajectories of individual simulations are presented in (H) and the distribution of the
1278 Antp isoforms, predicted by the model, in (I). (K-L') Model predictions (K and L) and
1279 experimental data validation (K' and L') of variability (K) and protein Fano factor (L) as
1280 a function of Antp concentration.

1281

1282 **Figure 6: Response of *Antp* to genetic perturbations.** (A-B) Overexpression of
1283 SynthAntp-eGFP long or short linker isoform result in tarsal transformations of the
1284 antenna (A), but normal leg development (B). These flies are fully viable and can be
1285 maintained as a stock. (C-C') Antp concentration and variability, measured by FCS, in
1286 leg discs of second and third instar larvae upon SynthAntp-eGFP long or short linker
1287 isoform expression. Despite persistent high concentration of Antp due to
1288 overexpression, variability is reduced. (D-I) Model response upon overexpression of
1289 Antp activating or repressing isoforms (similar to Fig. 5H-J). (J) Overexpression of an
1290 exogenous repressor (Scr) results in abnormal distal leg development, bearing
1291 malformations of the tarsus and femur. (K-N) Similar to (C-I) (see also Supplemental
1292 Figure S13E-I'). Antp concentration and variability, measured by FCS in the proximal
1293 leg disc of second (early) and third (late) instar larvae upon overexpression of
1294 mCherry-SynthScr.