regulate precise levels of *cog-1* gene expression, thus necessitating an additional level of gene expression control mediated by a miRNA. The elucidation of mechanisms that restrict *lsy-6* expression to just one of two bilaterally symmetrical taste neurons will provide further insights into the molecular mechanisms of establishing left/right asymmetry. □

Methods

Wild-type and mutant strains

We used the following nematode strains: wild-type N2 variation Bristol, CB4856 Hawaiian wild-type isolate, OH2535: *lsy-6(ot71)*, OH153: *cog-1(ot28)*, and OH1445: *cog-1(ot62)*/+; *otIs114*; *him-5(e1490)*.

Reporter transgenes

We used the following reporter transgenes: *ntIs1* Is[*gcy-5*^{prom}::*gfp*; *lin-15*(+)][3], *otIs3* Is[*gcy-7*^{prom}::*gfp*; *lin-15*(+)][3], *otIs114* Is[*lim-6*^{prom}::*gfp*; *rol-6*(*d*)][3], *otIs151* Is[*ceh-36*^{prom}::*rfp*; *rol-6*(*d*)], and *syIs63* Is[*cog-1*::*gfp*; *dpy-20*(+)][21]. (See Supplementary Information for a description of other transgenic lines.)

DNA construction and injection

All constructs were generated by polymerase chain reaction fusion[22]. A list of all constructs and primers used can be found in the Supplementary Information. DNA was injected at 2–20 ng μl^{-1} depending on the experiment (see Supplementary Information) with either *rol-6* (100 ng μl^{-1}) or *unc-122*::*gfp* (50 ng μl^{-1}) as the injection marker.

Scoring of phenotype

Animals were scored as adults. Quantification of defects shown in the figures can be found in the Supplementary Information.

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A self-organizing system of repressor gradients establishes segmental complexity in *Drosophila*

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Gradients of regulatory factors are essential for establishing precise patterns of gene expression during development[1–3]; however, it is not clear how patterning information in multiple gradients is integrated to generate complex body plans. Here we show that opposing gradients of two *Drosophila* transcriptional repressors, Hunchback (Hb) and Knirps (Kni), position several segments by differentially repressing two distinct regulatory regions (enhancers) of the pair-rule gene *even-skipped* (*eve*). Computational and *in vivo* analyses suggest that enhancer sensitivity to repression is controlled by the number and affinity of repressor-binding sites. Because the *kni* expression domain is positioned between two gradients of Hb, each enhancer directs expression of a pair of symmetrical stripes, one on each side of the *kni* domain. Thus, only two enhancers are required for the precise positioning of eight stripe borders (four stripes), or more than half of the whole *eve* pattern. Our results show that complex developmental expression patterns can be generated by simple repressor gradients. They also support the utility of computational analyses for defining and deciphering regulatory information contained in genomic DNA.

In *Drosophila*, the pair-rule gene *eve* is expressed in a pattern of seven stripes during the syncytial blastoderm stage of development. This pattern foreshadows the mature segmented body plan and is regulated by five enhancers[4–8]. Three enhancers drive expression of single stripes (*eve* 1, *eve* 2 and *eve* 5), and the remaining two drive expression of pairs of stripes (*eve* 3 + 7 and *eve* 4 + 6). The best characterized *eve* enhancer drives the expression of stripe 2 (*eve* 2)[9,10], which is activated in a broad anterior domain by the maternal morphogens Bicoid and Hb. Borders of the stripe are formed by repressive interactions involving the gap proteins Giant (Gt) and Kruppel (Kr), which are expressed in gradients anterior and posterior to the stripe, respectively. Activation and repression are mediated by the direct binding of all four proteins to discrete sites in the enhancer[9,11]. Thus, this enhancer acts as a transcriptional switch that senses activator/repressor ratios in individual nuclei.

Considerably less is known about the molecular regulation of the enhancers that drive two stripes. *eve* 3 + 7 is activated by ubiquitous factors including dSTAT92E[12,13], and activation of *eve* 4 + 6 requires the function of the *fish-hook* gene[14], but other activators are unknown. Genetic studies showed that the gap genes *hb* and *kni* are required for forming the borders of all four of these stripes. *kni* is

expressed in a broad posterior domain located between *eve* stripes 4 and 6 (Fig. 1a). In *kni* mutants, the two-stripe patterns driven by *eve* 3 + 7–*lacZ* and *eve* 4 + 6–*lacZ* reporter genes are completely derepressed in the region between the stripes[6,8]. By contrast, *hb* is expressed in an anterior domain that abuts *eve* 3 and a broad posterior stripe that overlaps *eve* 7 (Fig. 1b). In zygotic *hb* mutants, there are marked derepressions of the outer borders of the stripes driven by both the *eve* 3 + 7 and *eve* 4 + 6 reporter genes[6,8].

To test whether the *eve* 3 + 7 and *eve* 4 + 6 enhancers are differentially sensitive to Kni- and Hb-mediated repression, we used the *snail*(*sna*) promoter to misexpress these genes along the ventral surface of the embryo (Fig. 1f, k). The ectopic domain directed by this promoter is uniformly distributed along the anterior–posterior axis[15], and forms a ventral to dorsal gradient of protein diffusion (data not shown). As all seven *eve* stripes are subject to the same increase in protein concentration, differential sensitivities among stripes can be assayed directly. Weakly affected stripes will be repressed only in the ventral-most nuclei, whereas strongly affected stripes will show repression in more lateral or even dorsal regions.

Ventral expression of either Kni (*sna:kni*) or Hb (*sna:hb*) is sufficient for repression of *eve* stripes 3, 4, 6 and 7 in ventral regions

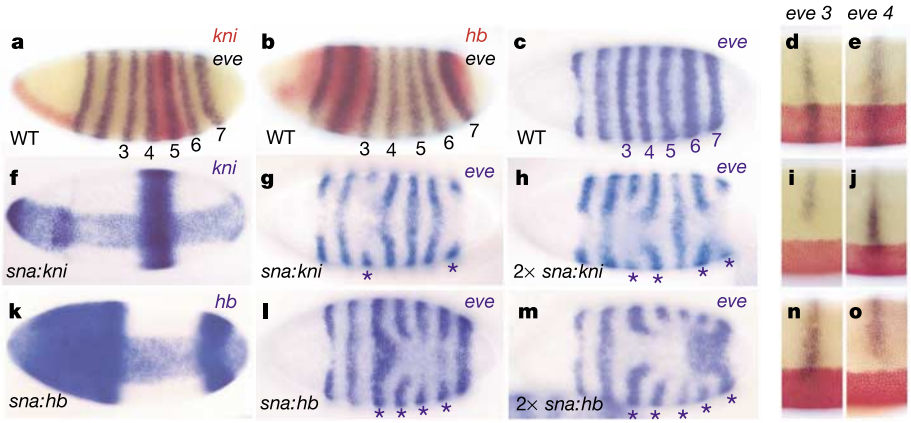

Figure 1 Individual *eve* stripes are differentially responsive to gradients of Kni and Hb. **a**–**e**, mRNA expression patterns in wild-type embryos at mid-cleavage cycle 14. **a**, **b**, Lateral views showing the spatial relationships between *hb*, *kni* and *eve*. **c**, Ventral view of the expression pattern of *eve*. Stripes 3–7 are numbered. **d**, **e**, Lateral views of embryos carrying the *eve* 3 + 7–*lacZ* (**d**) or the *eve* 4 + 6–*lacZ* (**e**) transgenes. Only the regions of stripe 3 or stripe 4 (black) are shown. Embryos are also stained to detect the expression pattern of endogenous *sna* (red). **f**–**o**, Expression patterns in embryos containing an ectopic domain of Kni (**f**–**j**) or Hb (**k**–**o**) expression along the ventral embryonic surface. **f**–**h**, **k**–**m**, Ventral views showing stripe-specific repression (compare with **c**). Affected stripes are marked by asterisks. **i**, **j**, **n**, **o**, Lateral views of embryos carrying the *eve* 3 + 7–*lacZ* (**i**, **n**) or the *eve* 4 + 6–*lacZ* transgene (**j**, **o**), in addition to the *sna:kni* or *sna:hb* misexpression constructs (compare with **d** and **e**).

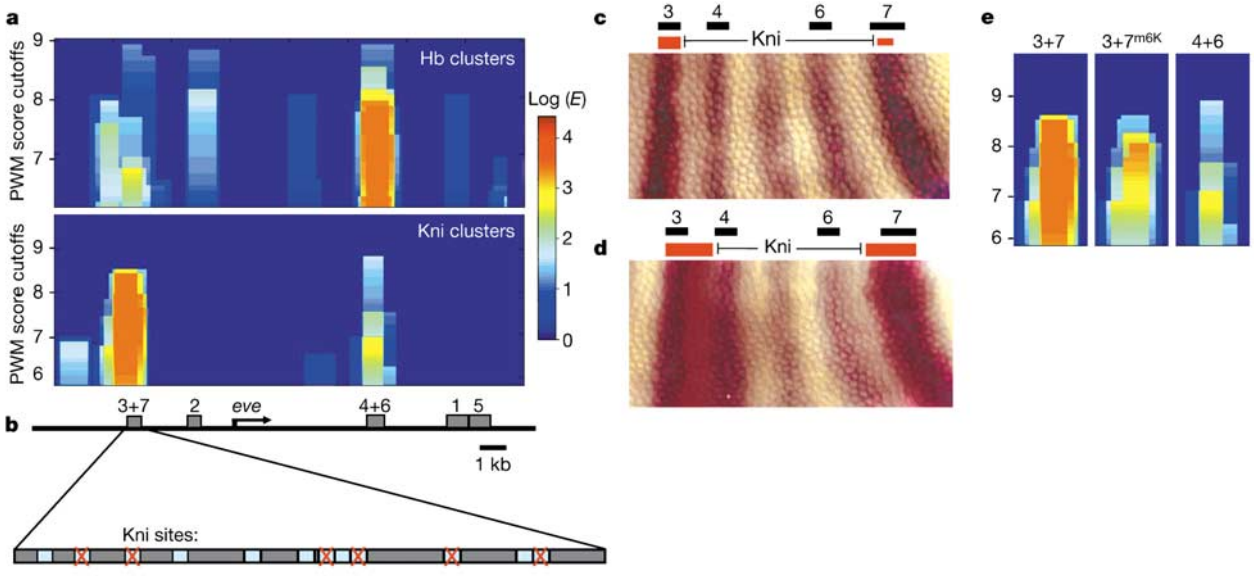

Figure 2 Clusters of repressor-binding sites determine enhancer sensitivity. **a**, Computer-predicted Hb- and Kni-binding site clusters in the *eve* locus. The heights of the bars along the *y* axis represent the binding affinities (PWM values) of the clustered sites. Site density is represented by a colour code (right). The positions of the clusters in the *eve* locus are represented along the *x* axis. **b**, The 500-bp minimal *eve* 3 + 7 enhancer contains 12 predicted Kni-binding sites. Six of these sites (X) were mutated to reduce their PWM value to close to zero (3 + 7m6K; see Supplementary Table 1). **c**, **d**, Expression of endogenous *eve* mRNA (black) compared with *lacZ* reporter mRNA (red) driven by the wild-type 3 + 7 enhancer (**c**) and the mutant 3 + 7m6K enhancer (**d**). Note the derepression of the 3 + 7m6K–*lacZ* reporter as compared with 3 + 7–*lacZ*. **e**, Mutating 6 of the 12 Kni sites in the 3 + 7 enhancer changes the apparent 'sensitivity' of the enhancer to one that is intermediate between the wild-type 3 + 7 and 4 + 6 enhancers.

(Fig. 1), but specific stripes require different quantities of ectopic protein for repression. One copy of the *sna:kni* transgene (1 × *sna:kni*) represses *eve* stripes 3 and 7, but has little effect on stripes 4 and 6 (Fig. 1g). Two copies repress all four stripes, but stripes 3 and 7 are more strongly repressed than stripes 4 and 6 (Fig. 1h). Misexpression of Hb shows the opposite effects. One copy of *sna:hb* causes a strong repression of stripes 4, 5 and 6, and an anterior weakening and posterior expansion of stripe 3 (Fig. 1l). The posterior expansion is probably caused by Hb-mediated repression of *kni* (see below).

Two copies of *sna:hb* cause a stronger repression of stripes 4, 5 and 6, repress stripe 3 completely in ventral-most nuclei, and considerably affect stripe 7, which seems slightly weaker and expanded anteriorly, again toward the region normally occupied by *kni* (Fig. 1m). The weaker effect on stripe 7 suggests that higher concentrations of Hb are required to repress this stripe. This is consistent with the fact that the posterior *hb* stripe overlaps stripe 7, and that additional factors (including Tll) are required for activation of this stripe[8]. The strong repressive effect of ectopic Hb on stripe 5 (Fig. 1l, m) is unexpected as this stripe seems to be normal in *hb* mutants[6]. In addition, computational analysis shows that there are very few Hb-binding sites in the *eve* 5 enhancer region (Fig. 2a). These results suggest that Hb-mediated repression of this stripe is indirect.

The above results suggest that the *eve* 3 + 7 and *eve* 4 + 6 enhancers respond autonomously to different amounts of the Hb and Kni repressors. To test this idea further, *lacZ* reporter genes driven by the minimal *eve* 3 + 7 or *eve* 4 + 6 enhancer were crossed into embryos carrying the *sna:kni* or *sna:hb* misexpression transgene (Fig. 1i, j, n, o). Embryos were also stained for endogenous *sna* expression, which forms a sharp ventral–lateral border, a landmark for measuring the extent of repression along the dorsal–ventral axis.

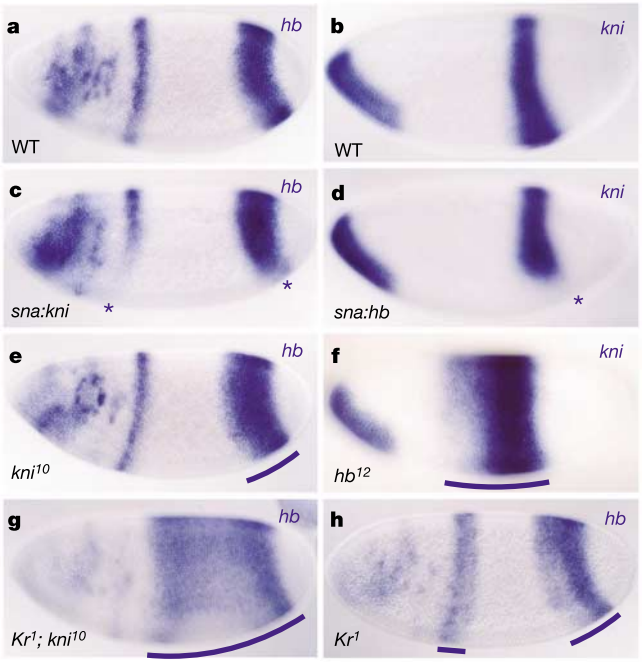

Figure 3 Mutual repression between Hb and Kni. **a**, *hb* mRNA expression in a wild-type embryo. **b**, *kni* mRNA expression in a wild-type embryo. **c**, *hb* mRNA expression in an embryo carrying one copy of the *sna:kni* transgene. **d**, *kni* mRNA expression in an embryo carrying one copy of the *sna:hb* transgene. **e**, *hb* mRNA expression in a kni^{10} mutant embryo. **f**, *kni* mRNA expression in a hb^{12} mutant embryo. **g**, *hb* mRNA expression in a Kr^{1} kni^{10} double mutant embryo. **h**, *hb* mRNA expression in a Kr^{1} mutant embryo. Repressive activities are indicated by asterisks, domain expansions are marked by lines below the embryos.

Ventral repression of the *eve* 3 + 7–*lacZ* transgene by Kni (2 × *sna:kni*) extends at least five nuclei above the *sna* border, but the *eve* 4 + 6–*lacZ* transgene is repressed only within the *sna* domain (Fig. 1i, j). Ventral expression of Hb (1 × *sna:hb*) causes the opposite effects: the *eve* 4 + 6–*lacZ* transgene is more strongly repressed than *eve* 3 + 7–*lacZ* (Fig. 1n, o). These experiments are consistent with the effects observed for the endogenous *eve* stripes.

To determine how these enhancers sense differences in repressor concentration, we used bioinformatics to analyse the distribution and affinity of Hb- and Kni-binding sites in the *eve* locus. Position-weighted matrices (PWMs) for each protein were generated by compiling and aligning the sequences of all known Hb- and Kni-binding sites[16], and a clustering algorithm[17] was used to search the 20-kilobase (kb) region surrounding the *eve* locus. This analysis identified only two main clusters for each factor in this region, which overlap precisely with the positions of the *eve* 3 + 7 and 4 + 6 enhancers (Fig. 2a). The composition of sites within these clusters, however, is very different. The 3 + 7 enhancer contains considerably more Kni sites with higher PWM scores than does the 4 + 6 enhancer (Supplementary Figs 1 and 2), consistent with its higher sensitivity to repression by Kni.

For Hb, searching with a low PWM cutoff value (>4.0) identified 11 sites in the more sensitive 4 + 6 enhancer and, unexpectedly, 16 sites in the 3 + 7 enhancer. These results are similar to previous findings[18]; however, 10 of the 11 Hb sites in the 4 + 6 enhancer have very high PWM scores (>6.3), as compared with only 6 in the 3 + 7 enhancer. Also, six of the ten high-scoring sites in the 4 + 6 enhancer are very tightly clustered in a 130-base-pair (bp) interval, whereas those in the 3 + 7 enhancer are evenly distributed across the sequence. These results suggest that binding-site affinity and distribution may be crucial parameters in determining enhancer sensitivity to Hb-mediated repression.

We next tested whether the clustering algorithm could predictably change enhancer sensitivity, using the Kni-binding sites in the 3 + 7 enhancer as a test case. The PWM search identified 12 Kni-binding sites in the minimal 3 + 7 enhancer; six of these sites were mutated (Supplementary Table 1) so that the cluster significance score of the mutated enhancer (denoted $3 + 7^{m6K}$) was intermediate between those of the wild-type 3 + 7 and 4 + 6 enhancers (Fig. 2b, e). Reporter expression driven by $3 + 7^{m6K}$ shows a derepression of the inner borders of stripes 3 and 7, suggesting that Kni-mediated repression has been compromised by these mutations. The stripe 3 response of the mutated enhancer extends throughout the inter-stripe region posterior to *eve* 3 to the anterior border of, but not through the region occupied by, *eve* 4 (Fig. 2, compare c and d). Thus, the $3 + 7^{m6K}$ enhancer is less sensitive to Kni than is the wild-

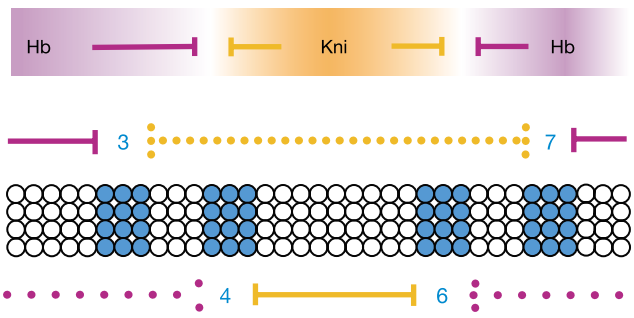

Figure 4 Repressor gradients and the generation of pattern complexity. Mutual repression between the Hb and Kni gradients fixes the positions of, and spacing between, their repressor gradients. These gradients are then 'read' by two enhancers that are differentially sensitive to repression by Hb or Kni, which ensures that *eve* stripes 3, 4, 6 and 7 are expressed at precisely defined positions.

type 3 + 7 enhancer, but is still more sensitive than the 4 + 6 enhancer. This suggests that the precise positioning of these stripes is controlled by the 'strength' of Kni site clusters.

As the normal Hb and Kni gradients set several expression boundaries in the region between their domains, it is essential that their relative positions in the embryo are precisely established and maintained. This could be achieved by mutual repression, as suggested by previous studies[2,19,20]. To test this further, we analysed the effects of ventrally expressed Kni on the expression of *hb* messenger RNA, and vice versa. Misexpression of Kni causes a strong reduction in *hb* mRNA in ventral regions (compare Fig. 3a and Fig. 3c). Similarly, misexpressed Hb causes a strong repression of *kni* (compare Fig. 3b and Fig. 3d).

Loss-of-function experiments lend further weight to the mutual repression hypothesis. In *hb* mutants, there is a substantial expansion of the posterior *kni* domain (Fig. 3f). In *kni* mutants, there is a slight anterior expansion of the posterior *hb* domain, but no effect on the anterior domain. Double mutant embryos that lack *kni* and the central gap gene *Kruppel* (*Kr*) show, however, a marked expansion of zygotic *hb* expression throughout the posterior half of the embryo (Fig. 3e, g, h). Because misexpression of Kr alone has no effect on the *hb* expression pattern (data not shown), this observation suggests that Kr and Kni may cooperate in repression of *hb*.

In conclusion, we have demonstrated the principle elements of a simple repression system that greatly increases pattern complexity in the *Drosophila* embryo. Strong reciprocal repression between *kni* and *hb* positions a symmetrical Kni domain between two opposing gradients of Hb (Fig. 4). This arrangement permits a single enhancer to make two stripes, one on both sides of the Kni domain. Two differentially sensitive enhancers effectively double the patterning information in each gradient, leading to the establishment of eight expression boundaries. A similar antagonistic relationship exists between the gap genes *gt* and *Kr*, which are expressed in non-overlapping domains, with the central *Kr* domain positioned between two *gt* domains[21]. The *eve* 2 and *eve* 5 stripes are formed on either side of the *Kr* domain by Kr- and Gt-mediated repression, but in this case each stripe is regulated by a separate enhancer, probably because the activators of these stripes are expressed in localized patterns[6,7].

Previous studies have shown that activator gradients are crucial for differential positioning of target gene expression patterns along the anterior–posterior and dorsal–ventral axes[3,22,23]. Our results and other studies[2,24] suggest that repressor gradients can also specify several gene expression boundaries by interacting with differentially sensitive regulatory elements. At the molecular level, repression mechanisms are flexible: enhancer activation can be prevented by direct repression or by interfering with the binding or activity of even a single activator protein[25,26]. We propose that repressor gradients, owing to this flexibility, are inherently more effective than activator gradients at providing developmental patterning information.

The sensitivity of an enhancer is likely to be determined by several parameters including the number, affinity and arrangement of repressor-binding sites, but predicting the relative importance of each of these parameters for a given enhancer is difficult. For the Kni repressor gradient, the different responses of the 3 + 7 and 4 + 6 enhancers seem to depend on different numbers of binding sites. By contrast, the different responses of the same enhancers to Hb repression seem to depend on the affinity and/or arrangement of sites. Thus, it may be impossible to formulate simple rules that describe the functional characteristics of most enhancers. However, future studies that combine computational analyses with experimental tests will undoubtedly increase our ability to identify and to characterize the genomic elements that regulate transcription. □

Methods

Fly stocks

We used the following amorphic alleles: kni^{10}, hb^{12} and Kr^{1}. The *eve* 4 + 6–*lacZ* line (PC N40C52-D[6]) was a gift from M. Fujioka and J. Jaynes. The *eve* 3 + 7–*lacZ* line used here contains the 500-bp minimal *eve* 3 + 7 enhancer and has been described[8].

Ventral expression of Kni and Hb

To make the *sna:kni* transgene, the *knirps* coding sequence and *eve* 3′ untranslated region (UTR) were excised as an *Asp*718–*Xba*I fragment from construct 22FKE[19], blunt-ended and cloned into the *Pme*I site of *pCas:sna* (ref. 27), a gift from L. Andrioli. For *sna:hb*, a 2.4-kilobase (kb) *Nde*1–*Eco*RI *hb* complementary DNA fragment was fused to an 0.8-kb *Eco*RI–*Xba*I fragment from the *α-tubulin* 3′ UTR, blunt-ended and cloned into the *Pme*I site of *pCas-sna*. We generated 7 and 15 independent lines with the *sna:hb* and *sna:kni* constructs, respectively, as described[28]. All misexpression lines tested generated quantities of ectopic protein that were considerably lower than endogenous Hb and Kni.

The *sna:hb* and *sna:kni* constructs contain transcriptional stop signals flanked by FRT sites downstream of the *sna* promoter to prevent ectopic expression during production and maintenance of transgenic lines. To activate ventral expression, males carrying a *β2-tubulin–FLP* transgene[29] and *sna:hb* or *sna:kni* were crossed to *yw* virgins, or those carrying the *eve* 3 + 7–*lacZ* or *eve* 4 + 6–*lacZ* transgenes. Embryos aged 2–4 h were collected and analysed by *in situ* hybridization as described[28].

Mutagenesis of the *eve* 3 + 7–*lacZ* reporter construct

Kni-binding sites in the *eve* 3 + 7 enhancer were mutated by site-directed mutagenesis of single-stranded DNA using a pBS-3 + 7NSKS construct as a template. We used the following oligomers: N2, 5′-CAAAAAACTGATCTAGCTAGCTAATGCAGCGAG-3′; N3, 5′-GCTGGGAAATGGCTAGGCGGCCATAAACCG-3′; N8 + 9, 5′-GCGCACAATGGC TAGAAAAAACGTGATCTACCTAGCTAATACGGGCG-3′; N10, 5′-CGCTGGGTTC GGGCTAGAAAACTAGCGCAG-3′; N11, 5′-CAAACACAAACAAACGCTTGTTAAAAA CGAGAGC-3′.

The mutant enhancer ($3 + 7^{m6K}$) was cloned into *pCaSpeR–eve–lacZ*, which contains the *eve* basal promoter, the *lacZ* cDNA and the *α-tubulin* 3′ UTR[30]. The sequence of the minimal *eve* 3 + 7 enhancer showing the positions of all predicted Kni and Hb sites is provided as Supplementary Fig. 1. Four lines containing the $3 + 7^{m6K}$–*lacZ* construct were generated and tested. All gave similar results.

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The gene product Murr1 restricts HIV-1 replication in resting CD4$^+$ lymphocytes

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Although human immunodeficiency virus-1 (HIV-1) infects quiescent and proliferating CD4$^+$ lymphocytes, the virus replicates poorly in resting T cells[1–6]. Factors that block viral replication in these cells might help to prolong the asymptomatic phase of HIV infection[7]; however, the molecular mechanisms that control this process are not fully understood. Here we show that Murr1, a gene product known previously for its involvement in copper regulation[8,9], inhibits HIV-1 growth in unstimulated CD4$^+$ T cells. This inhibition was mediated in part through its ability to inhibit basal and cytokine-stimulated nuclear factor (NF)-κB activity. Knockdown of Murr1 increased NF-κB activity and decreased IκB-α concentrations by facilitating phospho-IκB-α degradation by the proteasome. Murr1 was detected in CD4$^+$ T cells, and RNA-mediated interference of Murr1 in primary resting CD4$^+$ lymphocytes increased HIV-1 replication. Through its effects on the proteasome, Murr1 acts as a genetic restriction factor that inhibits HIV-1 replication in lymphocytes, which could contribute to the regulation of asymptomatic HIV infection and the progression of AIDS.

Murr1 is a highly conserved 190-amino-acid protein that does not have any identifiable motifs, and a homozygous deletion in the gene encoding canine Murr1 leads to copper toxicosis in Bedlington terriers[8]. In this study, Murr1 was initially identified in a two-hybrid screen by binding the X-linked inhibitor of apoptosis, a known activator of NF-κB (refs 10, 11, and E.B., unpublished observations). To study its effect on NF-κB, HIV-1 reporter plasmids with wild-type or mutant (ΔκB) sites[2] were co-transfected with control or Murr1 expression plasmids in the different cell lines. Murr1 inhibited both basal and tumour necrosis factor (TNF)-α-dependent HIV-1 transcription from the wild-type but not the κB-mutant reporter in Jurkat T-leukaemia and 293T renal epithelial cell lines (Fig. 1a, left and middle panels). In contrast, Murr1 did not substantially inhibit tumour growth factor-β-dependent transcription in HepG2 cells, confirming its specificity (Fig. 1a, right panel). The κB effect was dose-dependent and observed with other inducers of NF-κB, including interleukin-1 (IL-1) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Fig. 1b). Murr1 modulated the expression of endogenous κB-regulated genes: transfection into 293T cells decreased the endogenous cell-surface expression of major histocompatibility complex (MHC) class I, in contrast to CD9, which is independent of NF-κB (Fig. 1c).

Its site of action in the NF-κB signalling pathway was further defined by co-transfection of different regulators with an NF-κB reporter in Jurkat T cells. Whereas Murr1 inhibited both IKK-1- and IKK-2-induced NF-κB activity (Fig. 1d, middle and right panels), it failed to block RelA-mediated transcription (Fig. 1d, left panel), indicating that Murr1 might interact downstream of the IκB kinase signalosome. As determined by immunoprecipitation, co-transfected haemagglutinin (HA)-tagged Murr1 and Myc-tagged IKK-2 did not associate *in vivo* (Fig. 2a, lane 2, left panel). Although IKK-1 also did not associate with Murr1 (data not shown), an interaction between transfected HA-tagged Murr1 and endogenous IκB-α was readily detected (Fig. 2a, lane 6). The ankyrin domain of IκB-α was required for association with Murr1, as were amino acids 1–160 of Murr1 (Supplementary Fig. 1a).

A polyclonal antibody against Murr1 demonstrated the association between endogenous Murr1 and IκB-α *in vivo*. RelA antibody immunoprecipitated IκB-α, IκB-β and Murr1 (Fig. 2b, lane 10). IκB-α antibody also pulled down RelA and Murr1 (Fig. 2b, lane 12), but the IκB-β antibody did not precipitate Murr1 (Fig. 2b, lane 14), suggesting that Murr1 interacted preferentially with the NF-κB–IκB-α complex. This association was confirmed *in vivo* by confocal microscopy with fluorescent fusion proteins (Supplementary Fig. 1b), similarly to the pattern of RelA association with IκB-α[12–14].

The physiological consequences of these interactions were determined by knockdown of endogenous Murr1 in 293T cells using control and Murr1-specific short interfering RNA (siRNA) duplexes. The specificity of two such siRNAs, Murr1-1 and Murr1-2, directed to different Murr1 sequences, was first confirmed by transfecting 293T cells with wild-type or mutant siRNAs along with wild-type or mutant Murr1 complementary DNAs modified at the siRNA target site (Supplementary Fig. 2). Transient transfection of Murr1-specific siRNA duplexes downregulated endogenous Murr1 and IκB-α, had little effect on IκB-β, p65 or IKK-2 (Fig. 3a, left panel), and increased κB-dependent reporter activity (Fig. 3a, right panel).

To investigate the mechanism of Murr1 action, 293T cells were transfected with a control or Murr1 siRNA. Four days after transfection, cells were treated with the proteasome inhibitor MG132 for 2 h or with vehicle alone and stimulated with TNF-α. Cells depleted of Murr1 showed a decrease in basal IκB-α (Fig. 3a) and an increase and persistence of phospho-IκB-α in response to stimulation with TNF-α (Fig. 3b, left panel). This effect was observed in the absence of a proteasome inhibitor, MG132, but not in its presence (Fig. 3b, right panel), indicating that Murr1