LETTERS

The microRNA *miR-196* acts upstream of Hoxb8 and Shh in limb development

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MicroRNAs (miRNAs) are an abundant class of gene regulatory molecules (reviewed in refs 1, 2). Although computational work indicates that miRNAs repress more than a third of human genes[3], their roles in vertebrate development are only now beginning to be determined. Here we show that *miR-196* acts upstream of Hoxb8 and Sonic hedgehog (Shh) *in vivo* in the context of limb development, thereby identifying a previously observed but uncharacterized inhibitory activity that operates specifically in the hindlimb. Our data indicate that *miR-196* functions in a fail-safe mechanism to assure the fidelity of expression domains that are primarily regulated at the transcriptional level, supporting the idea that many vertebrate miRNAs may function as a secondary level of gene regulation.

Sonic hedgehog (Shh) is a key signal mediating anterioposterior polarity in both the fore- and hindlimb buds[4]. Retinoic acid (RA) signalling is required for Shh expression in the forelimb and the hindlimb[5–8]. The transcription factor Hoxb8 seems to mediate the induction of Shh by RA in the forelimb in that Hoxb8 is upregulated as an immediate-early response to ectopic RA administered to the chick forelimb bud[7], and ectopic Hoxb8 expression in the anterior of the forelimb of a transgenic mouse leads to Shh expression[9]. Ectopic RA does not lead to Hoxb8 induction in the hindlimb bud, however, owing to the presence of an unknown hindlimb-specific inhibitory activity[10].

Reasoning that the unknown hindlimb inhibitory activity[10] might be mediated by a small silencing RNA, we blocked miRNA processing by using a conditional knockout allele of Dicer, a key enzyme required for producing functional miRNAs from their precursors[11,12]. Dicer activity can be specifically removed from the limb buds by using a conditional allele[13] and a limb-specific *Prx1::cre* construct[14] (Supplementary Fig. 1a), which recombine floxed alleles efficiently in the limb mesenchyme (Supplementary Fig. 1b). To test whether the inhibition of Hoxb8 induction by RA in hindlimb buds is relieved by the removal of Dicer activity, hindlimbs from $Dicer^{\Delta floxed/\Delta floxed}$ and wild-type mice at embryonic day 11.5 (E11.5) were cultured in the presence of RA. As in chick limbs, the presence of RA led to a marked upregulation of *Hoxb8* messenger RNA in the forelimb tissue of both wild-type and mutant animals (Fig. 1a, b), but not in wild-type hindlimbs (Fig. 1c). In $Dicer^{\Delta floxed/\Delta floxed}$ hindlimbs, however, RA induced the expression of *Hoxb8* (Fig. 1d). As previously shown[13], loss of Dicer activity does not affect the expression of other known patterning genes in the developing limb bud (Supplementary Fig. 1c). Thus, the previously uncharacterized inhibitory activity[10] is lost in the absence of Dicer.

Dicer is crucial for the processing of hundreds of miRNAs and many siRNAs. To identify specific candidate miRNAs that could be responsible for the hindlimb-specific inhibitory activity downstream of Dicer, we used microarray analysis[15]. Of the miRNAs that are expressed in the limb primordia, 12 were at least twofold more abundant in either the forelimb or the hindlimb bud (Fig. 2a and Supplementary Table 1). The most differentially expressed miRNA in the screen was *miR-196*, with an expression signal in the hindlimb exceeding by 20-fold that in the forelimb (Fig. 2a and Supplementary Table 1). Differential *miR-196* expression was verified in northern blot analyses of RNA isolated from forelimbs and hindlimbs of both chick and mouse (Fig. 2b) and was also consistent with the expression domain suggested by a transgenic reporter study[16]. Intriguingly, *Hoxb8* mRNA is a known target of *miR-196 in vivo*[16,17]. Therefore, we investigated whether *miR-196* might be the unknown hindlimb-specific activity preventing *Hoxb8* induction by RA.

First, to establish that *Hoxb8* is indeed an *in vivo* target of *miR-196* in the hindlimb, we carried out a modified 5′ rapid amplification of

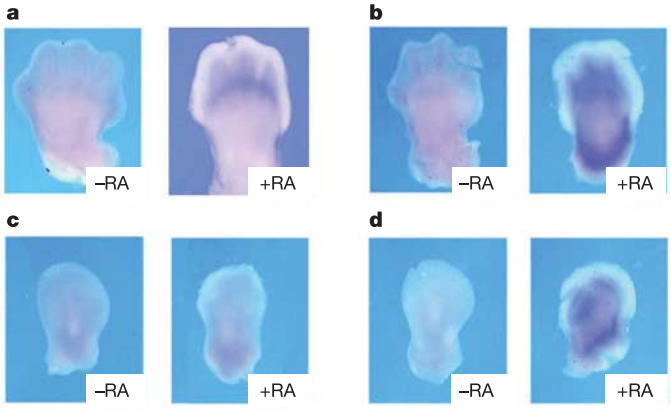

Figure 1 | Activity downstream of Dicer inhibits RA-induced expression of *Hoxb8* in mouse hindlimbs. a, E11.5 $Dicer^{floxed/+}$;*Prx1::Cre* (wild-type) forelimbs were cultured without RA (−RA), leading to no detection of *Hoxb8* ($n = 6/6$), or with 100 nM RA for 12 h (+RA), leading to induction of *Hoxb8* ($n = 6/6$). Expression of *Hoxb8* was detected by means of whole-mount *in situ* hybridization. **b**, E11.5 $Dicer^{floxed}/Dicer^{floxed}$;*Prx1::Cre* (*Dicer* knockout) forelimbs subjected to the same treatment similarly resulted in induction of *Hoxb8* only in the presence of RA ($n = 6/6$ negative, without RA; 6/6 positive, with RA). **c**, Hindlimbs from the mice in **a** were cultured similarly, and RA failed to induce *Hoxb8* expression ($n = 8/8$ with RA; 8/8 without RA). **d**, Hindlimbs from the *Dicer* knockout mice in **b** were cultured similarly. Complete deletion of *Dicer* did not result in induction of *Hoxb8* in untreated hindlimbs ($n = 5/5$), but it enabled the accumulation of *Hoxb8* transcripts in RA-treated hindlimb mesenchyme ($n = 6/6$).

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complementary DNA ends (RACE) protocol, commonly used as an assay for miRNA-directed mRNA cleavage[17,18]. By sequencing the 5′ RACE products, we could determine whether any amplified *Hoxb8* degradation products were cleaved precisely at the predicted *miR-196*-binding site. We could easily observe *miR-196*-directed *Hoxb8* cleavage in the wild-type hindlimb, whereas *Hoxb8* cleavage in the forelimb tissue was barely seen (Fig. 3a, b). These data indicate that *Hoxb8* is indeed both transcribed, at a level detectable by polymerase chain reaction (PCR), and cleaved *in vivo* in the hindlimb.

In wild-type chick embryo, after 2.5 d of incubation *Hoxb8* is expressed in the neural tube and somites. *Hoxb8* is also expressed in the forelimb field, where it functions in inducing *Shh* during the early limb field stages (Fig. 3c). To test whether *miR-196* activity could attenuate *Hoxb8* expression at the early limb field (stage 16), we used a replication-competent viral expression system (RCAS). Our analysis showed that 26 h after *in ovo* injection of the virus *RCAS::miR-196*, *Hoxb8* expression was reduced throughout the embryo and, in particular, endogenous expression of *Hoxb8* in the forelimb field was markedly repressed (Fig. 3d).

We next addressed whether *miR-196* could be responsible for the inability of ectopic RA to induce *Hoxb8* in the hindlimb[10]. We implanted RA-soaked beads into wild-type chick forelimbs, which induced *Hoxb8* within 4 h (Fig. 3e). By contrast, parallel implantations failed (or were only marginally able) to induce *Hoxb8* in forelimb buds ectopically expressing *miR-196* (Fig. 3f). Misexpression of *miR-196* in the forelimb thus creates a situation that is reminiscent of wild-type hindlimb, in which endogenously high expression of *miR-196* leads to observable degradation of endogenous *Hoxb8* and correlates with an inability of RA to induce ectopic *Hoxb8*.

The *miR-196*-sensitivity of *Hoxb8* thus provides a compelling explanation for the inability of RA to induce *Hoxb8* in the hindlimb. In previous studies[7,10], RA and Hoxb8 were placed upstream of Shh expression in the forelimb and, indeed, blocking endogenous RA activity resulted in a significant, albeit incomplete, downregulation of endogenous Shh expression[7,10]. If the *miR-196*-sensitivity of Hoxb8 expression were truly involved in mediating RA-induced expression of Shh in the forelimb bud, then Shh expression itself should be downregulated on the introduction of *miR-196* into the forelimb. Indeed, when chick embryos were analysed 2 d after viral misexpression of *miR-196* in the right limb field, endogenous *Shh* was consistently downregulated (Fig. 4a, compare with 4b). Other genes, not described to be downstream of *Hoxb8* in the limb mesenchyme, were not affected by misexpression of *miR-196*, suggesting that this was a specific effect (Supplementary Fig. 2). To quantify the effect of *miR-196* on *Shh* levels, we infected chick embryos as above and assayed them 2 d later by quantitative real-time PCR. *Shh* expression was decreased in the *miR-196*-infected forelimb to roughly a third of the level seen in wild-type limbs (Fig. 4c).

We also checked whether ectopic misexpression of *miR-196* would block RA-induced ectopic expression of *Shh*. When RA-soaked beads were implanted into wild-type chick forelimb for 36 h, an anterior domain of ectopic Shh was induced[4] (Fig. 4d); however, in *miR-196*-infected limbs, *Shh* expression was blocked or diminished and more diffuse (Fig. 4e). Although *Shh* was repressed by *miR-196* misexpression in the forelimb, the expression of *Shh* in the hindlimb was not affected by the same manipulation (Fig. 4f, g). This difference highlights the rather unexpected conclusion that independent pathways control *Shh* expression in the forelimb and the hindlimb (Fig. 4h), which may be explained by a dual role for Hox genes in specifying forelimb versus hindlimb identity and in regulating Shh expression. After *Hoxb8* and other related Hox genes evolved to specify forelimb-specific morphology, a different, *Hoxb8*-independent, mechanism of regulating *Shh* downstream of RA had to evolve for the hindlimb.

Despite the evidence presented here and elsewhere[9] supporting a role for *Hoxb8* in regulating *Shh* in the forelimb, it has been reported that even the removal of all three Hox8 paralogues has no effect on limb formation[19], suggesting that this gene has possible redundancy with other Hox genes. In this respect, *Hoxa7* is also expressed in the posterior of the forelimb bud and is induced by RA[20,21]. Moreover, we found that, like *Hoxb8*, *Hoxa7* is expressed in a forelimb-specific fashion (Supplementary Fig. 3). Intriguingly, *Hoxa7* is also a predicted target of *miR-196*, with several conserved matches to the 5′ portion of the miRNA known as the 'seed'[22]. We did not observe changes in *Hoxa7* mRNA in response to *miR-196* misexpression (data

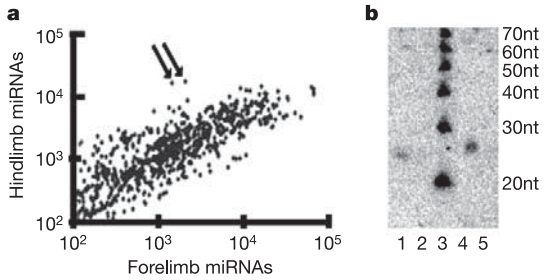

Figure 2 | Hindlimb-specific expression of *miR-196*. **a**, Representation of miRNA array analysis, comparing the expression of individual miRNAs (dots) in E10.5 mouse forelimb and hindlimb buds (in arbitrary units). Abundance of an individual miRNA in the hind- and forelimb is shown by its relative position along the logarithmically scaled *y* and *x* axes, respectively. Arrows indicate features corresponding to *miR-196*. **b**, Northern blot hybridization detected *miR-196* in extracts from hindlimbs of E10.5 mouse and stage-22 chick (lanes 1 and 4, respectively) but not in mouse and chick forelimb buds (lanes 2 and 5, respectively). Data are representative of four independent samples. The lengths of DNA oligomers (lane 3) used as size markers are specified next to the blot in nucleotides (nt).

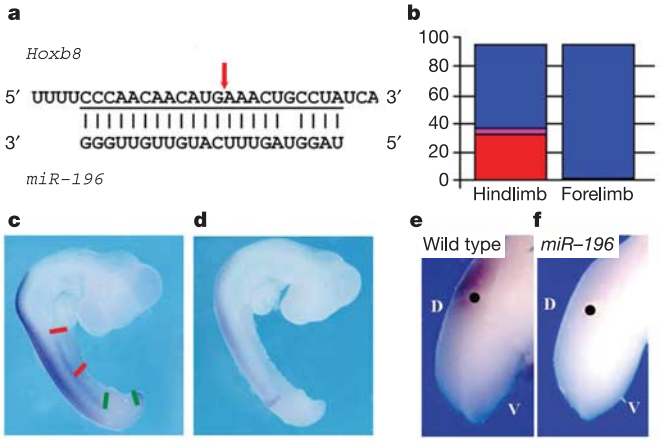

Figure 3 | *miR-196* downregulates *Hoxb8* accumulation. **a**, Sequence of the 3′ UTR of *Hoxb8* complements *miR-196*. An arrow indicates the 5′ end of the primary cleavage product. **b**, 5′ RACE analysis in hindlimb and forelimb. Of the 96 hindlimb clones sequenced, 33 yielded a sequence consistent with *miR-196*-directed cleavage (red); four were also truncated *Hoxb8* clones, but cleavage was outside the miRNA-binding site (pink); and 59 were sequences unrelated to *Hoxb8* (blue). In the forelimb, no clones were consistent with *miR-196*-directed cleavage. **c**, By whole-mount *in situ* hybridization with a *Hoxb8* probe, an expression domain of *Hoxb8* was detected in the forelimb field (red bars), but not in the hindlimb field (green bars), of a stage-16 chick embryo ($n = 8/8$). **d**, Early pan infection with *RCAS::miR-196* resulted in downregulation of *Hoxb8* ($n = 6/6$). **e**, An RA-soaked bead implanted into the anterior aspect of a stage-22 wild-type forelimb induced *Hoxb8* expression ($n = 8/10$). **f**, Only marginal induction of *Hoxb8* expression was detected on implantation of an RA-soaked bead in a forelimb infected with *RCAS::miR-196* ($n = 6/8$). Anterior view; D, dorsal; V, ventral.

not shown), however, indicating that if *miR-196* is repressing Hoxa7, it is reducing Hoxa7 protein without substantially destabilizing the *Hoxa7* transcript. Such a mechanism would be consistent with the results of a heterologous reporter assay showing that a *Hoxa7* untranslated region (UTR) fragment containing the *miR-196* seed matches predominantly mediates *miR-196*-dependent repression through the reduction of protein rather than mRNA levels[17].

The experiments described here indicate that *miR-196* may be an *in vivo* inhibitor of *Hoxb8* in the hindlimb, and thereby may be responsible for the inability of ectopic RA to induce *Hoxb8* in the hindlimb. Low *Hoxb8* expression and *miR-196*-directed degradation was detected in the naive hindlimb bud by 5′ RACE, indicating that *miR-196* activity is a component of *Hoxb8* regulation in the unmanipulated limb. Notably, however, loss of miRNA activity in the Dicer-deficient hindlimb did not, in itself, result in *Hoxb8* induction, suggesting that the primary level of regulation of forelimb-specific *Hoxb8* expression is transcriptional and independent of small regulatory RNAs.

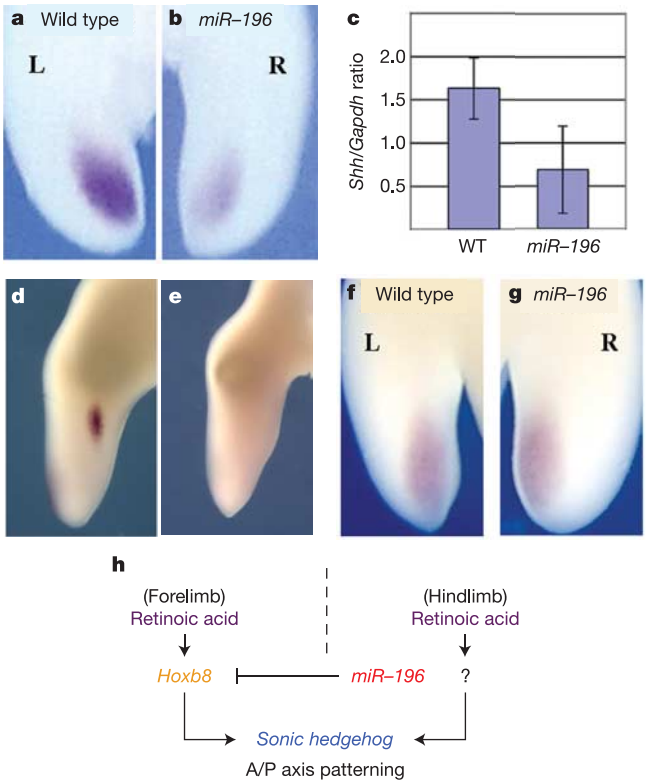

Figure 4 | *miR-196* downregulates *Shh* in the chick forelimb. **a**, Expression of *Shh* in the left (L) limb of stage-23 embryo. Posterior view ($n = 20/20$). **b**, In the right forelimb (R) of the same embryo, endogenous *Shh* expression was diminished 2 d after infection with *RCAS::miR-196* ($n = 18/20$). **c**, Three untreated sample tubes, each containing four forelimbs (stage 23), and three corresponding sample tubes with *RCAS::miR-196*-infected limbs were subjected to real-time PCR quantification of *Shh* mRNA. Three replicate runs were done on each sample tube. Blue bars represent the expression of *Shh*, normalized to *Gapdh*, in wild-type limbs (mean ± s.d., 1.62 ± 0.35) and limbs infected with *RCAS::miR-196* (0.68 ± 0.51). The difference in the mean value between the *miR-196*-infected sample and the untreated control was significant (one-tailed *t*-test, $P = 0.029$). **d**, One and a half days after an RA-soaked bead (1 mg ml^{-1}) was implanted into the anterior aspect of stage-20 forelimbs, an ectopic *Shh* expression domain was detected by whole-mount *in situ* hybridization ($n = 6/6$). **e**, *RCAS::miR196* infection inhibited the ectopic expression of Shh in the anterior ($n = 5/8$). **f**, **g**, *Shh* expression was comparable in the left uninfected hindlimb (**f**) and the right *RCAS::miR196*-infected hindlimb (**g**) of chick embryos ($n = 20/20$). **h**, Model of the epistatic relations among *miR-196*, RA, *Hoxb8* and *Shh* in the developing limbs. A/P, anterior–posterior.

Thus, in normal limb development, the role of *miR-196* seems to be to safeguard against inappropriate Hox activity in the hindlimb. This conclusion fits well with the report that the genes that are downregulated when a miRNA is delivered to human cells are preferentially those that are expressed at low levels in tissues that normally express the miRNA[23]. It thus seems that a chief role of some miRNAs in vertebrate development may be to prevent inappropriate activity of genes in domains where they are already repressed transcriptionally. Some miRNAs have been experimentally implicated to have roles in other facets of vertebrate development, including *miR-181* in haematopoiesis[24], *miR-430* in brain morphogenesis[25] and *miR-1* in heart development[26]. In contrast to our findings, *miR-1* and its target *hand2* are predominantly expressed in the same cells, enabling *miR-1* to have a key role in regulating the switch between cardiomyocyte differentiation and proliferation[26]. Together, these two studies indicate that these intriguing regulators of gene activity can take on diverse roles in coordinating vertebrate developmental and physiological processes.

METHODS

Mice and organ culture. Mice were housed and handled in accordance with protocols approved by the Institutional Animal Care and Use Committee of Harvard Medical School. Male mice carrying one copy of the *Prx1::Cre* allele and one *Dicer*^{floxed} allele were crossed to *Dicer*$^{floxed/floxed}$ females. Cre recombinase, driven by the *prx1* enhancer, excises a required region in the RNAse IIIb domain to yield a nonfunctional *Dicer* allele in limb buds[13]. Timed-pregnant females were killed at E11.5, embryos were dissected, and limbs were separately cultured in hanging drops. After 12 h of incubation in DMEM medium supplemented with 10% fetal calf serum, penicillin and streptomycin with or without 100 nM all-*trans* RA (Sigma), limbs were fixed in 4% paraformaldehyde for 4 h and processed for *Hoxb8 in situ* hybridization.

MicroRNA–cDNA probe and expression array hybridization. Total RNA was isolated from E10.5 mouse fore- and hindlimbs with Trizol (Invitrogen) according to the manufacturer's instructions. Small RNAs were size-fractionated, ligated to adaptor oligonucleotides, reverse-transcribed and amplified. Labelled probes (Cy5 for the hindlimb sample and Cy3 for the forelimb sample) were hybridized to an expression array as described[15]. After hybridization, the array was scanned (Genepix pro 4000b; Axon) and analysed. Along with the vertebrate spots on the array, spots for all known *Caenorhabditis elegans* miRNAs are printed, most of which should not be hybridized to a vertebrate probe. Thus, background was set at a score equal to 95% that of the spots from the *C. elegans* section of the array[15].

5′ RACE of *Hoxb8*. Total RNA was obtained from a pool of 30 E10.5–11 mouse hind- and forelimbs and was subjected to modified 5′ RACE as described[17] with the following primers: 5′-CCATAAAGCAATTCACAGATACAGG-3′ and 5′-GGTTGCGAGGAAAGATG-3′.

Generation of *RCAS::miR-196*. A 500-bp fragment of genomic DNA surrounding the chicken *miR-196-1* locus (chromosome 27, *HoxB* cluster) was amplified by PCR. An *Apa*I site was appended to the 5′ end and an *Eco*RI site was appended to the 3′ end by using the following primers (restriction sites are in parentheses): 5′-AATTCC(GGGCCC)CTCTATTTGTCAACTATTTGTAACG-3′ and 5′-G(GAATTC)GCATTTTGGCCTCCGAGAGG-3′. The PCR fragment was then cloned, by means of the *Apa*I and *Eco*RI sites, downstream of the RNA polymerase III U6 promoter, into a pBS–U6 plasmid. The whole U6 promoter and *miR-196* genomic DNA were then excised with *Cla*I and cloned into the RCAS virus. *RCAS::miR-196* viral particles at a titre of 10^{10} particles per ml were collected from the medium of transfected chicken embryonic fibroblasts. Proper transcription and processing of mature *miR-196-1* was confirmed by northern blots of total RNA extracted from chicken embryonic fibroblasts (data not shown).

Chicken embryo manipulations and *in situ* hybridization. Fertilized eggs were obtained from SPAFAS and incubated at 37 °C, and the embryos were staged according to ref. 27. Eggs were incubated up to stage 7–8 and then the whole embryo was targeted by multiple injections of *RCAS::miR-196*. Alternatively, at stage 12–13 the coelomic cavity was targeted to infect the lateral plate mesoderm. Resin beads were soaked in 100 nM all-*trans* RA in dimethylsulphoxide for 1 h and then implanted into the anterior of stage-22 chick forelimbs for a further 4 h, as described[10], except that AG-1X8 beads (Bio-Rad) were used. Alternatively, RA-soaked AG-1X2 beads (1 mg ml^{-1} = 300 mM) were implanted into stage-20 limbs that were allowed to develop *in ovo* for 36 h more[4]. Embryos were then collected and fixed in 4% paraformaldehyde overnight. Whole-mount *in situ* hybridization and probes have been described[4,28]. The *Hoxa7* probe was

amplified directly by PCR from chicken genomic DNA and transcribed, without subcloning, by using the following primers: 5′-ACCTACACCCGCTACCAGAC-3′ and 5′-TGTAATACGACTCACTATAGGGCCCTCTTCCTCATCTTCTTCCA-3′.

Quantitative real-time PCR for chick *Shh*. Three untreated sample tubes, each containing four stage-23 forelimbs, and three corresponding sample tubes with *miR-196*-infected limbs were subject to quantification of *Shh* mRNA. Three replicate runs were done on each sample tube with a Lightcycler 2000 (Roche) using SYBER Green DNA Master Mix (Roche) and the following primers: GAPDH-5′, 5′-CGGAGTCAACGGATTT-3′; GAPDH-3′, 5′-ATAACACGCTTA GCACC-3′; Shh-5′, 5′-TGCTAGGGATCGGTGGATAG-3′; Shh-3′, 5′-ACAA GTCAGCCCAGAGGAGA-3′. A 'no RT' control was done in parallel (data not shown). One-tailed *t*-test determined the significance of the difference in the mean value between the *miR-196*-infected sample and the untreated control.

Received 24 June; accepted 10 August 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank E. McGlinn and S. Nissim for critically reading the manuscript. This work was funded by grants from the NIH (to C.J.T. and to D.P.B.). E.H. was supported by a 'Dorot' fellowship and 'Bikura' award. J.H.M. is supported by a Kirchstein postdoctoral fellowship.

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