The wild-type Rev-ErbA/ROR response element and mutant Rev-ErbA/ROR response elements were constructed as follows: the following oligonucleotide constructs were inserted into the Smal site of the SV40 3′-leader vector1. WT 5′-GATGTTGCGAAATAGTTGTTGGTGCGACATTTAGGGAAGGCAGAAAGTAGGTCAGGGACGGAGG-3. Mutant 5′-GATGTTGCGGAAGCTTCTAGGTCTCAGACATTTAGGGAAGGCAGAAAGTAGGTCAGGGACGGAGG-3. The constructs were verified by sequencing.

Transfection and real-time monitoring of circadian bioluminescence
Rat1-R12 cells (ATCC) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Sigma). Cells were plated at 1 × 10⁵ cells per dish in 35-mm dishes 24 h before transfection. Cells were transfected with LipofectAMINE 2000 reagent (GIBCO BRL) according to the manufacturer's instructions. The cells in each dish were transfected with 1 μg (total) of expression plasmids. After 16 h, cells in each dish were treated with 0.1 μM dexamethasone (Sigma), and after 2 h these media were replaced with 10% fetal bovine serum (Sigma) supplemented with 10 μM HEPES (pH 7.2), 0.1 mM luciferin (Promega), and antibiotics (25 U ml⁻¹ penicillin, 25 μg ml⁻¹ streptomycin). Bioluminescence was measured with photomultiplier tube (PMT) detector assemblies (Hamamatsu). The modules and cultures were maintained in a light-tight incubator at 36 °C and interfaced to IBM PC-type computers for continuous data acquisition. The PMT was positioned about 2 cm above the culture, and photon counts were integrated over 1-min intervals.

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the distal limb would be lost after removal of AER at early as opposed to late stages. Thus, AER removal could produce the observed pattern of distal truncation without reference to the progress zone or other models of proximodistal specification.

To test for this possibility, we collected wing buds 6–8 h after AER removal at various stages and assayed for apoptotic cells using a TdT-mediated dUTP nick end labelling (TUNEL) assay. Our results confirmed previous reports of cell death in a region extending up to 200 μm from the apex after AER removal at stage 22, and verified the previous report that cell death is no longer detectable 10 h after removal of AER (data not shown). Moreover, when we removed the AER between stages 18 and 22, the region of cell death remained similar in size (Fig. 1 and data not shown; n = 3–5 per stage). At later stages, however, progressively less cell death was observed, with the region displaying significant cell death restricted distally by stage 24 (Fig. 1 and data not shown; n = 3). After stage 25, no cell death was detected (data not shown; n = 3).

Thus, although rapid cell death could potentially account for the progressive truncations seen after AER removal at early and mid stages of limb development, it cannot explain truncations within the autopod (the most distal skeletal segment of the limb) when the AER is removed late in limb development. However, these late stage truncations could be explained if signals from the AER also affect proliferation of distal limb bud cells. To test this hypothesis, we assayed for cell proliferation by measuring 5-bromodeoxyuridine (BrdU) incorporation after AER removal. Indeed, we saw a marked loss of BrdU incorporation in the distal 200 μm of mesenchyme within 8 h after removing the AER from stage 24 wing buds (Fig. 1 and Table 1; n = 3).

In spite of these observations, the question remained whether the combination of cell death and decreased cell proliferation would be sufficient to explain the truncations seen after AER removal. We tested this by labelling distal cells and observing their fate after AER removal. At stage 19, only the stylopod (future humerus) forms after AER extirpation. According to the progress zone model, such truncations, which supposedly result from an arrest in the autonomous specification process thought to function within the progress zone, should allow for normal incorporation of labelled distal cells into the forming stylopod. On the other hand, if truncations arise because distal cells die, then distally labelled cells should mostly be absent after AER removal. Stage 19/20 (n = 25) and stage 23 (n = 12) limb buds were injected with Dil at 100 μm from the AER, and as a control, DiO was injected 300 μm below the AER. After a 24-h incubation, expansion of both proximal and distal cell populations was observed in control limbs containing an intact AER (Fig. 2a, c). In contrast, only a small number of labelled distal cells were found to survive after AER removal, and those that did remained tightly localized, whereas the proximal cell population expanded both in number and in distribution within the limb bud (Fig. 2b, d).

Furthermore, when embryos that had undergone removal of the AER at stage 19 (n = 12) and stage 23 (n = 9) were allowed to incubate for 4 days, sections through these limbs demonstrated that proximal cells, but not distal cells, contributed to skeletal structures (Fig. 2e, f). These experiments demonstrate that distal cells do not contribute to skeletogenesis after AER removal. Thus, observing skeletal pattern after AER removal does not provide information regarding the specification of the distal cells at the time of surgery. Moreover, these data suggest that cell loss has a causal role in the resultant distal limb truncations.

Previous experiments have shown that application of fibroblast growth factor (FGF) protein can rescue proximodistal patterning when applied immediately after removal of the AER6,7. Notably, FGF protein also prevents cell death in the distal limb bud mesenchyme after AER removal7. If these two results are causally related, then application of FGF protein after cells have died should fail to rescue truncations in AER-deleted early limb buds. Conversely, as a decrease in proliferation could be reversible, some distal pattern might be restored after a similar delay in FGF application when the AER is removed at later stages. To test this, we removed the AER and stapled heparin beads soaked in FGF4 to the distal tip of the operated limb, either immediately or after incubating the embryos an additional 12 h to allow for completion of cell death5. At stage 19/20, when appreciable levels of cell death are seen after AER removal,

Table 1 Reduction in BrdU incorporation in the distal 200 μm of limb bud

<table>
<thead>
<tr>
<th>Embryo Treatment</th>
<th>n</th>
<th>BrdU positive nuclei (%)</th>
<th>Fold reduction†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Intact AER</td>
<td>6,033</td>
<td>35.4 (2.7)</td>
<td></td>
</tr>
<tr>
<td>2 Removed AER</td>
<td>5,920</td>
<td>17.0 (3.6)</td>
<td>2.1</td>
</tr>
<tr>
<td>3 Intact AER</td>
<td>8,220</td>
<td>40.3 (5.4)</td>
<td></td>
</tr>
<tr>
<td>4 Removed AER</td>
<td>8,900</td>
<td>14.3 (2.4)</td>
<td>2.8</td>
</tr>
<tr>
<td>5 Intact AER</td>
<td>7,097</td>
<td>41.0 (3.6)</td>
<td></td>
</tr>
<tr>
<td>6 Removed AER</td>
<td>9,069</td>
<td>13.2 (4.0)</td>
<td>3.1</td>
</tr>
</tbody>
</table>

Measurements were taken 8 h after removal of the AER at stage 24. Numbers in parentheses indicate standard deviation.

†(Per cent positive nuclei for intact AER)/(per cent positive nuclei for removed AER).
Embryos were injected with both DiI (red) and DiO (green), and the fluorescent lipophilic dye DiI labelled the autopod (Fig. 3a–f). Wing buds of embryos were injected with the fluorescent lipophilic dye DiI and incubated for 3 days. a–f, Wing buds of stage 20 (a, b, e) or stage 23 (c, d, f) embryos were injected with both DiI (red) and DiO (green), 100 μm and 300 μm below the AER, respectively. After 20 h of incubation, both the proximal and distal cell populations expand in the unoperated limb buds (a, c); however, after AER removal, only the proximal cell population continues to expand significantly whereas the distal population fails to expand (b, d). Although some distal cells survive after AER removal (e, f), they do not incorporate into the limb skeleton after 4 days of development. Arrowheads mark incorporation of DiO into developing cartilage.

Cell death is responsible for limb deletions after AER removal. g–i. Cell death is responsible for limb truncations after AER removal at early stages. After extirpation of the AER at stage 19 (g, i), heparin beads soaked in FGF4 were stapled to the distal limb bud (h). When FGF4 protein is added immediately after extirpation of the AER at either stage, a complete proximodistal set of skeletal elements develops (j). However, after the wave of cell death, FGF4 signalling can only rescue development in limbs of later stage embryos (compare k with l). The arrowhead in l denotes rudimentary digits. In each panel, distal is to the right. Magnification is 10× (a–f) or 4× (g–l).

FGF4 was able to rescue the proximodistal pattern only when added immediately after AER removal (Fig. 2g, i; n = 14). However, when the experiment was performed at stage 23, when less cell death is observed, FGF signalling was able to rescue proximodistal patterning defects even when applied 12 h after AER removal (Fig. 2h, j, l; n = 8).

Taken together, these data demonstrate that the domain of cell death is constant to a depth of approximately 200 μm after removal of the AER at early stages of limb development; there is a substantial decrease in cell proliferation within the distal mesenchyme after AER removal at all limb bud stages analysed; and these effects result in loss of distal structures. Notably, examination of the most detailed fate maps available1 shows that removal of progenitor cells in the distal 200 μm of the limb bud at various stages results in precisely the amount of distal deletion observed after extirpation of the AER at each of these stages. Therefore, we propose that the final skeletal pattern observed after AER removal does not reflect the level of patterning achieved through a particular stage of development. Rather, it reflects the proportion of progenitor cells which are outside the range of signals produced by the AER and are therefore not affected by the changes in cell death and proliferation caused by removal of the AER. Our data further emphasize the importance of the AER, and by extension FGFs, in preventing cell death and maintaining cell proliferation, as opposed to maintaining a cell autonomous patterning clock.

As the AER extirpation experiments do not give a reliable estimate of when proximodistal segments are specified during limb development, we turned to alternative approaches to address this issue. In a first set of experiments, we used the lipophilic dye Dil to examine the fates of cells in the early forelimb bud. Groups of neighbouring cells within a region less than 33 μm in diameter were

Figure 2 Cell death and decreased cell proliferation are significant factors in phenotypes produced by AER removal. a–f, Wing buds of stage 20 (a, b, e) or stage 23 (c, d, f) embryos were injected with both Dil (red) and Dio (green), 100 μm and 300 μm below the AER, respectively. After 20 h of incubation, both the proximal and distal cell populations expand in the unoperated limb buds (a, c); however, after AER removal, only the proximal cell population continues to expand significantly whereas the distal population fails to expand (b, d). Although some distal cells survive after AER removal (e, f), they do not incorporate into the limb skeleton after 4 days of development. Arrowheads mark incorporation of Dio into developing cartilage.

Figure 3 Descendants of cells in the early limb bud contribute to only one proximodistal segment. Wing buds of embryos were injected with the fluorescent lipophilic dye Dil and incubated for 3 days. a–c, Injections at the level of somite 18 directly below the AER labelled the autopod (c), injections 100–200 μm below marked the zeugopod (b) and injections 200–300 μm below marked the stylopod (a). d–g, Expansion of segment progenitors occurs in a proximal-to-distal sequence. Notably, Dil label applied at stage 19 extends the length of the respective segment, suggesting that expansion of the individual progenitor pools has not been completed by stage 19. However, Dil injections 200–300 μm below the AER at stage 20 yield a discrete spot of label 3 days later, indicating that the stylopod progenitor population stops expanding by stage 20 (d), whereas the zeugopod progenitors are still expanding at stage 22 (e) but not by stage 23 (g). By contrast, injections subjacent to the AER at stage 26 result in label that extends throughout the phalanges, indicating that autopod progenitors have not finished expanding by stage 26 (f). Magnification is 4×.
labelled with Dil at stage 19 and assayed 3–4 days later. In most of the injections, labelled cells did not appear in more than one skeletal segment (Fig. 3a–c and Table 2; n = 228 out of 244). Thus, cells labelled directly under the AER marked the autopod (future digits), those labelled 100 μm beneath the AER marked the zeugopod (future radius/ulna), and those labelled 200 μm below the AER marked the stylopod. In a small number of cases, label was present in adjacent segments or was localized to the segmental boundary (n = 16 out of 244). We interpret these as representing cases where the injection site was at the border of two future segments and cells on both sides of the border were labelled. We also found that cells at the extreme anterior and posterior margins of the limb bud crossed the boundaries between proximodistal segments (data not shown), as has been noted previously. However, as these cells are not part of the chondrogenic region, it is not obvious that this finding is relevant to the discussion here.

Although Dil injections at different proximodistal levels at stage 19 were almost always confined to single limb segments, it was striking that these labelled cell populations consistently extended throughout the entire length of the segment (Fig. 3a–c). Thus, at stage 19, there is considerable mixing of cells along the proximodistal axis within each future segment, but not between segments. This suggests that the three limb segments are already specified and have different cellular properties at this early stage.

The spread of Dil in these limbs indicates that progenitor populations in all three segments undergo considerable expansion after injection. In contrast, at stage 20, we found that labelling cells with Dil at 250 μm below the AER resulted in a discrete spot of label confined to the stylopod but significantly smaller than the full limb segment (Fig. 3d; n = 2 out of 18 expanded). Thus, the stylopod progenitor cell population completes its expansion during the roughly 8 h between stage 19 and stage 20, consistent with the results of a previous report suggesting that proximal cell populations have fully expanded by stage 20. Similarly, we found that expansion of the zeugopod terminates between stages 22 (Fig. 3e; n = 11 out of 12 expanded) and 23 (Fig. 3g; n = 2 out of 12 expanded), whereas expansion of the autopod continues past stage 26 (Fig. 3i; n = 6 out of 9 expanded). In an accompanying manuscript, Sun et al. describe a model for AER-FGF function based on the concept that expansion of the stylopod, zeugopod and autopod progenitor populations is completed in such a proximal-to-distal sequence.

To test directly whether the distal cells of the limb bud are specified to form autopod by stage 19, as the Dil labelling experiments would suggest, we transplanted the distal portion of stage 19 wing buds (fated to be autopod) to other sites in the embryo. When

| Table 2 Proximodistal fate of cells Dil-labelled at various positions at stage 19 |
|----------------------------------------|------------------|--|--|--|--|--|--|
| Position (μm) | Wing-Bud segment† | | | | | | |
| U | Autopod | Zeugopod | Stylopod | Other† |
| 17 | 25 22 3 | | | |
| 18 | 34 2 | | | |
| 19 | 31 29 2 | | | |
| 100 | 18 24 1 | 2 | 21 | | |
| 19 | 17 2 | | | |
| 200 | 12 | | | |
| 18 | 19 | | | |
| 19 | 21 | | | |
| 300 | 11 | | | |
| 18 | 10 | | | |
| 19 | 16 | | | |

†Distance from the apex of the wing bud to the injection site. U, area directly underlying the AER. †Somite opposite the injection site. ‡Number of limbs injected per injection site.

Figure 4 Distal cell fates are already specified but not determined in the early limb bud. a, Posteriorly biased slices from the distal tip of stage 19 wing buds approximately 100 μm thick were grafted to the stump of a donor leg bud. b, After 6 days of incubation, cartilage development was analysed by staining with alcian blue. Only digit-like elements formed from these grafts. The arrowhead denotes the graft–host interface. To verify that the transplanted cells maintained distal specification, some transplants were collected after 2–3 days, fixed and embedded in paraffin. c, d, In situ hybridization on 10-μm sections revealed that the population of transplanted cells, marked by wing-specific expression of Hoxa-13 (c), correlates with expression of Hoxa-13 (d), a marker of the future autopod. e, To test when distal cells are determined to be digits, a 75–100-μm slice of distal mesenchyme from the anterior two-thirds of the limb bud was disaggregated, re-aggregated, packed into an ectodermal hull and grafted to an embryo. After additional development, recombinant limbs were collected and the skeletal structure visualized using Victoria blue stain. f, g, Recombinant limbs generated from the entire anterior mesenchyme of stage 20 limbs (f) form all three proximodistal segments, as do limbs consisting of only distal anterior mesenchyme from stage 19 embryos (g). h–j, By stage 22, recombinants of distal mesenchyme form only the zeugopod and digits (h), whereas stage 23 recombinants are restricted to form digits (i), and stage 24 recombinants generate exclusively phalanges (j). Magnification is 4× (b, f–j) or RELSP10× (c, d).
stage 19 distal wing tips were transplanted onto the stump of an amputated leg bud (Fig. 4a, b; n = 9) or into the coelom (data not shown; n = 6), the graft formed only digit-like skeletal elements. To verify the distal specification of the transplanted tissue, we examined molecular markers. Because a piece of the wing bud was grafted to the leg bud, tissue derived from the transplant could be unambiguously identified 2–3 days after transplantation using the wing-specific marker Tbx5 (refs 11, 12) (Fig. 4c). In every case, the entirety of the graft expressed the autopod-specific marker Hoxa-13 (ref. 13) (Fig. 4d; n = 5).

On the basis of our Dil labelling and transplantation experiments, we conclude that the proximodistal axis is patterned quite early in normal development. However, these studies do not address when cells of the various limb segments become determined, that is, refractory to re-specification to a different proximodistal fate under experimental conditions. Indeed, there is ample evidence indicating that early limb bud cells are not yet determined and therefore can change their proximodistal specification when challenged. For example, when limb mesenchyme is dissociated, re-aggregated, placed within an ectodermal hull, and then grafted back to a host embryo, the recombinant limb bud will develop and form skeletal structures14–16. Although the posterior limb bud must be excluded because mixing polarizing cells with the recombinant mesoderm has a negative effect on its further development17,18, if only the anterior two-thirds of the limb mesenchyme is used, all three limb segments can form relatively normal structures19 (Fig. 4f). Moreover, as cells from different proximodistal levels do not sort to their original position after re-aggregation15, the randomized organization of the recombinant limbs implies that cells formerly specified to form particular proximodistal structures contribute to different limb segments after this series of manipulations. We decided to use this model to examine when limb bud cells become determined with respect to their specific proximodistal fates.

When the entire stage-20 anterior limb bud mesenchyme was reassociated and allowed to develop, all three limb segments formed (Fig. 4f; n = 12 out of 12). We repeated this experiment using just the distal-most 75–100 μm of the anterior limb mesenchyme as a source of cells for the recombinant limb buds (Fig. 4e). A normal number of limb bud cells was achieved by pooling cells dissociated from the distal portion of multiple stage 20 limb buds. Importantly, these cells are already specified to form autopod structures according to the data described above. Moreover, according to the progress zone model, the distal limb bud cells have undergone significant proliferation between stages 17 and 20 and would be expected to have progressed beyond the specification of the stylopod. Nonetheless, recombinants made exclusively from the distal mesenchyme produce all three limb segments (Fig. 4g; 3 segments, n = 6 out of 9; 2 segments, n = 3 out of 9), showing that the distal cells are not yet determined and can be re-specified to form stylopod and zeugopod, as well as autopod.

At later stages, there is a progressive restriction in the ability of distal limb mesenchyme to produce proximal structures. Thus, at stage 22, recombinants formed zeugopod and digits but never a stylopod (Fig. 4h; 2 segments, n = 1 out of 2; 1 segment, n = 1 out of 2). Furthermore, at stage 23, recombinants were restricted in their competence to form autopod, including both metacarpals and phalanges (Fig. 4i; n = 6 out of 6). By stage 24, the distal mesenchyme only formed phalanges when re-aggregated (Fig. 4j; n = 5 out of 5). Similar results were seen at stage 26 (data not shown; n = 4 out of 4). Thus, there is a progressive restriction in the distal limb mesenchyme such that the cells are increasingly determined to form a narrower range of distal fates. This process of progressive determination takes place between stages 20 and 24.

In principle, the reaggregation data could also fit a progress zone model for specification if the distalization clock simply does not start ticking until stage 20. There is no reason to think this hypothetical clock would be inert during the earliest stages of limb proliferation and then suddenly start after stage 20, but once one accepts the old AER-removal specification map as flawed (owing to cell death), then the timing of this process is totally unclear. It seems less ad hoc to consider specification of cellular properties reflected in proximodistal pattern, and the timing of determination, when those properties can no longer be altered, to be distinct and separable events. Proximodistal determination, or the stage when cells are no longer capable of being re-specified along this axis, is relevant not only to the re-aggregation experiments reported here, but also to a variety of other experimental manipulations that similarly lead to re-specification of limb mesenchyme, including grafting an ectopic AER proximally or transplanting a polarizing region anteriorly at different stages. All of these test the capacity of mesenchymal cells to be re-patterned along the proximodistal axis at various developmental stages, not the timing when that pattern is normally laid down.

Similarly, the demonstration by ref. 20 that X-ray irradiation of limb buds causes a more severe effect on proximal than distal limb development has been viewed as evidence for progressive specification of proximodistal pattern. However, the implications of this experiment have been misunderstood because it has been interpreted in terms of the progress zone model of proximodistal specification when, more accurately, it addresses the degree to which the mesenchyme is determined at the time of irradiation. The progressive determination of distal fates is important as it limits the regulative ability of the undifferentiated tissue in the developing limb. In addition, differentiation of tissues within the limb proceeds in a proximodistal sequence such that, in the case of the wing, tissues of the shoulder and upper arm differentiate first5, whereas cells at the tip remain undifferentiated until later21. We suggest that these two properties, the progressive determination of undifferentiated cells and the proximal-to-distal wave of differentiation, together limit the ability of the developing limb to reconstitute pattern after cellular loss, and hence are probably responsible for the types of malformations seen after teratogenic events such as experimental irradiation and thalidomide exposure.

In contrast to the progressive nature of proximodistal determination, we find no evidence for progressive specification of fates as defined by the progress zone model. Rather, our results indicate that cell fates are established within the early limb bud at a time and over a spatial scale consistent with standard modes of inductive intercellular signalling. The realization that proximodistal specification and determination are distinct events will facilitate future work on the molecular and genetic bases for proximodistal limb patterning.

**Methods**

**Embryo manipulation**

Fertilized chickens were obtained from Spafas and stored at 10 °C until use. To continue development, eggs were incubated at 38 °C in a non-rotating incubator. Eggs were windowed as previously described22. Embryos were staged according to ref. 23.

Before labelling cells with the fluorescent lipophilic dyes DiI and DiO (1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate and 3,3'-dioctadecyloxycarbocyanine perchlorate, catalogue number D-282 and D-275, respectively; Molecular Probes, 5 mg ml−1 in dimethylformamide) the AER of the wing bud was lightly stained by pipetting a small amount of a 0.01% (w/v) solution of nile blue sulphate in water against the distal tip of the limb bud. Dye was injected into wing buds in vivo using an Eppendorf pressure injector (Model 5242). Distance of the injection site from the AER was measured using a calibrated reticle on a Leica stereomicroscope. AER extirpation, if performed, occurred immediately after injection using tungsten needles. After incubation for 3–4 days, embryos were fixed in 1% paraformaldehyde/PBS, equilibrated in 30% sucrose/PBS, and photographed. Some embryos were subsequently embedded in OCT compound and sectioned on a cryostat. Digital images of fluorescent signal were taken on a Nikon Eclipse E1000 microscope using the Y2/Ey/E Texas red (DiI) and fluorescein isothiocyanate/HYQ (DiO) filter sets.

For transplants, a 100-μm sliver of distal tip was cut from stage 19 wing buds opposite somites 17–19. This fragment was transplanted to a leg bud transected approximately 100 μm distal to the flank. Amputation of the recipient leg bud close to the flank was necessary to avoid intercalary regeneration of zeugopod fates24. The distal fragment was attached to the leg bud using ‘U’ shaped staples made from platinum wire (Goodfellow Metals; diameter 0.025 inches). After transplant, embryos were kept at room temperature for 1 h before returning to the incubator. In some cases the distal tips were grafted to the
colo
d of stage 16/17 embryos by making a slit in the dorsal side of the embryo, between the prospective limb buds and lateral to the somites. The graft was inserted through the slit with the cut edge medial. Recombinant limbs were prepared as described, except that mesenchyme was derived from the distal-most 75–100 μm of the anterior two-thirds of the wing bud and that digestion with collagenase was omitted. Recombinant buds were grafted to the somites, allowed to develop for 7 days, and stained with Victoria blue as described.

To rescue limb development in the absence of an AER, heparin-acryl beads were incubated in 1 mg ml\(^{-1}\) FGF4 (a gift from V. Rosen) at room temperature for 1 h, then stored on ice until use. After removal of the AER, the two heparin beads were attached to the limb bud with platinum staples. One bead was placed at the posterior margin and the other just anterior to the first. After attachment of beads, embryos were kept at room temperature for 30 min before returning them to the incubator. Embryos were collected after 6 days of incubation and fixed in 4% paraformaldehyde. Skeletally were stained with 0.02% alizarin blue 8GK in 70% ethanol/30% acetic acid at 37 °C, then cleared in 0.5% KOH and stored in glycerol.

**Analysis of cell proliferation and death**

After AER removal, embryos were returned to the incubator for 6–8 h. In cases where cell proliferation was examined, 200 μl of 5 mg ml\(^{-1}\) BrdU (Sigma) in PBS was injected around the embryo after 7 h, and the egg was returned to the incubator for 60 min. BrdU were collected, fixed in 4% paraformaldehyde/PBS overnight at 4 °C, washed in PBS, dehydrated through an ethanol series and embedded in paraffin. Sections (6 μm) were then stained with TUNEL (3-aminoethylbenzothiazoline)-treated slides, and apoptotic cells were stained using the method of ref. 25, except that biotin-16-dUTP was replaced by fluorescein-12-dUTP to allow for direct detection of transferred nucleotides. BrdU- labelled cells were detected using an anti-BrdU monoclonal antibody (clone BU-33, Sigma) followed by a Cy-2 conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories). Slides for fluorescent microscopy were stained in 0.5 μg ml\(^{-1}\) 4,6-diamidino-2-phenylindole (D-1306; Molecular Probes) in PBS for 1 min before mounting in 80% glycerol.

**In situ hybridizations**

Both the whole mount and non-radioactive section in situ hybridizations were performed using standard protocols for whole-mount in situ hybridization. Details protocols are available on request.

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DNA sequence variants in specific genes or regions of the human genome are responsible for a variety of phenotypes such as disease risk or variable drug response. These variants can be investigated directly, or through their non-random associations with neighbouring markers (called linkage disequilibrium (LD)). Here we report measurement of LD along the complete sequence of human chromosome 22. Duplicate genotyping and analysis of 1,504 markers in Centre d’Etude du Polymorphisme Humain (CEPH) reference families at a median spacing of 15 kilobases (kb) reveals a highly variable pattern of LD along the chromosome, in which extensive regions of nearly complete LD up to 804 kb in length are interspersed with regions of little or no detectable LD. The LD patterns are replicated in a panel of unrelated UK Caucasians. There is a strong correlation between high LD and low recombination frequency in the extant genetic