Single cells can sense their position in a morphogen gradient


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Accepted 9 September; published on WWW 9 November 1999

SUMMARY

* Xenopus blastula cells show a morphogen-like response to activin by expressing different genes according to the concentration of activin to which they are exposed. To understand how cells recognize their position in a concentration gradient, it is essential to know whether each cell responds individually to activin concentration. An alternative idea, proposed by previous work, is that cells need to interact with their neighbours to generate a concentration-related response. To distinguish between these ideas, we have cultured blastula cells under conditions which provide different degrees of contact with other cells, allowing nil to maximum communication with their neighbours. The cultures include cells attached to fibronectin and those unattached. The cultures also include cells that have no contact with any cell except their clonal progeny, cells that have lateral contact to neighbouring cells, and cells that are completely enveloped by other cells in a reaggregate. We have used RNase protection and in situ hybridization to assay the expression of the activin-responsive *Xenopus* genes *Xbra*, *Xgsc*, *Xeomes*, *Xapod*, *Xchordin*, *Mix1*, *Xlim1* and *Cerberus*. We find no difference in gene expression between cells attached to fibronectin and those unattached on agarose. Most importantly, we find that cells respond to activin in a concentration-related way irrespective of their degree of contact with other cells. Therefore interaction among cells is not required for the interpretation of morphogen concentration, at least in the case of the early genes studied here. We conclude that isolated blastula cells can sense and respond individually to activin by expressing genes in a concentration-dependent way.

Key words: *Xenopus*, Activin, Morphogen gradient, Eomesodermin, Xbrachyury

INTRODUCTION

Activin, a member of the TGFβ family of signalling factors, is present as mRNA and protein in the early embryos of *Xenopus* (Asashima et al., 1991; Thomsen et al., 1990), and is a candidate for one of the molecules involved in the mesoderm-inducing induction that takes place a few hours after fertilization (Smith, 1993). Activin has attracted much interest in recent years, because it can activate genes in a concentration-dependent manner, thereby behaving like a morphogen. The identification of mature activin, its cloned receptors, at least some of its transduction molecules and several of its immediate response genes has enabled its concentration-related behaviour to be analyzed in more detail than that of any other vertebrate morphogen.

The concentration-dependent effects of activin were first established by Green and Smith (1990) using dissociated blastula cells of *Xenopus*; these were exposed to different concentrations of activin, reaggregated and found to express a range of different genes in a dose-dependent way (Green et al., 1992). It was later shown that an activin-loaded bead can cause waves of gene expression to spread out through a static population of undissected blastula cells, establishing the point that each cell can make a nil, low or high gene response to activin concentration (Gurdon et al., 1994). There is, however, a key question that needs to be answered before a full understanding of morphogen gradients is achieved. This asks whether a single cell can determine its position in a concentration gradient. Does it know its 'positional value' (Wolpert, 1969) in the absence of other cells? A much more complicated situation would exist if a cell can establish its position and determine gene expression only by reference to other cells in the same concentration gradient. Indeed exactly this last conclusion has been reached as a result of experiments on dissociated *Xenopus* blastula cells. Green et al. (1994) and Wilson and Melton (1994) have both argued that the major part of the concentration-dependent response does indeed require interactions among cells of the responding population. They confirmed the previous results of Green et al. (1992), that the choice of genes expressed at 17 hours after activin addition is related to the initial activin concentration, but found that this was not true of the response seen at 3 hours, when most genes showed a similar progressive dose response.

We describe here a more detailed investigation of the short-term response of cells to different activin concentrations. This is for two reasons. First, in the course of our work, we reached conclusions that differ significantly from those published. Second, there has been no analysis so far of gene response to morphogen concentration in single cells independently of their neighbours. We have cultured activin-treated cells in different configurations so as to provide increasing degrees of cell contact and potential communication after activin treatment,
and have assayed gene responses at the single cell level by in situ hybridization, as well as quantitatively by RNase protection. We conclude that each cell can individually assess different activin concentrations and can activate early genes independently of any cell communication. This clarifies and simplifies our understanding of the mode of action of a morphogen.

**MATERIALS AND METHODS**

**Cell dissociation and activin treatment**

Animal caps were removed from blastulae at about stage 8.5, and their cells dissociated by incubation in Modified Barth Saline (MBS; Gurdon, 1977) lacking Ca2+ and Mg2+, but supplemented with EDTA (0.5 mM) and with 0.1% bovine serum albumin (BSA, fraction V; Sigma), for 10-15 minutes. Cells were dispersed by gentle pipetting, incubated for 10 minutes in the desired concentration of activin protein, washed twice in the above dissociation medium (10 caps per 1 ml), and then washed once in normal (Ca2+ - and Mg2+ -containing) MBS with 0.5% BSA, before final resuspension in the desired volume of MBS containing 0.1% BSA and 3x the normal MBS concentration of Ca2+ (that is at 6.5 instead of the normal 2.2 mM Ca2+).

Activin was prepared as in Dyson and Gurdon (1998), and was unlabelled. The concentrations of activin used here are given as μl activin per 100 μl medium. This refers to a percentage dilution of our stock preparation, prepared from the medium of mRNA-injected oocytes. We have assayed our activin preparations for their ability to induce mesoderm in animal caps. As in our earlier work (McDowell et al., 1997), the concentration of activin in our oocyte medium is about 20 nM. After Sephadex purification it is about 7 nM. A 0.45% concentration (that induces Xbra) therefore corresponds to 30 pM. Green et al. (1992) used Xbra activation by activin at 3 units/ml, corresponding to 15 pM. Since we incubate our cells in activin solution for only 10 minutes, compared to the continuous incubation of Green et al. (1992), the efficacy of our activin treatment is comparable to theirs.

**Plating of cells on substrates and cell culture**

An agarose layer was prepared on Nuncron 35 mm plastic dishes, using LMP, ultrapure, Gibco agarose at 1% in MBS. Fibronectin was adsorbed onto BDH superfrost plus microscope slides by layering 2 ml of a 20 μg/ml solution of fibronectin (from bovine plasma, Sigma) in water onto each 25×75 mm slide. After incubation for 2-4 hours at 23°C in a humidified atmosphere, the fibronectin solution was washed off and replaced by 1 ml of culture medium. Cells were pipetted at the desired density onto the fibronectin-coated slides within 10 minutes of removal of the excess fibronectin solution.

The culture medium for all substrates and configurations of cells was MBS with 3x its normal Ca2+ concentration, 0.5% BSA and 1 μg/ml Gentamycin (Sigma). All cells were cultured in a humidified atmosphere at 19-23°C until control embryos had reached the desired stage (usually stage 10.5). For freezing, cells were removed from fibronectin substrates by incubation for about 5 minutes in MBS lacking Ca2+ and Mg2+, but containing 0.5 mM EDTA. Preparations were fixed for 1 hour in Memfa (Harland, 1991), transferred to 100% methanol, and kept at −20°C in methanol until ready for further processing.

**RNase protection**

This was carried out according to Ryan et al. (1996). Quantitation was achieved by use of a Fujifilm Phosphorimager and MacBAS 2.5 software.

**In situ hybridization and single cell density quantitation**

Cells were hybridized on fibronectin slides according to Lemaire and Gurdon (1994) with slight modifications as described by Butler et al. (1999). The most important of these is a reduction in the duration of proteinase K treatment from 30 to 12 minutes. We used Boehringer BM purple to visualize hybridized probes. The quantitation of spread cell in situ hybridization results was carried out on low density preparations (see Fig. 1C). A small circular window in the centre of a photomicroscope eyepiece was placed over a cell (at ×20 magnification), and the light meter reading recorded. A low light intensity and appropriate film speed setting on the photographic monitor provided readings of 5-6 for an intensely stained cell, with background values of about 1. The background values were subtracted in preparing the results shown in Fig. 6. Colour may have reached saturation in the most strongly stained cells, but if so this would only have reduced the magnitude of the effects seen in Fig. 6. It would not change the conclusions.

**Cell staining**

The cells shown in Fig. 1. C-E were stained with rhodamine-phalloidin (Molecular Probes R-415) to mark cytoskeletal actin, and TOTO-3 (Molecular Probes T-3604) to stain nuclear DNA.

**RESULTS**

**Configuration and lineage of cell cultures**

To test the importance of cell contact and cell communication we have grown activin-treated animal cap cells on agarose or fibronectin substrates at different densities. We have cultured cells either singly or as aggregates on agarose or fibronectin substrates. The efficacy of our activin treatment is comparable to theirs.

- The clonal progeny of a single cell or sister pair, rather than being a mixed colony resulting from two or more original cells that happened to be plated next to each other and could therefore interact. To test this we mixed unlabelled and rhodamine-labelled (RlDx) blastula cells in a ratio of 3:1, respectively, and made low density cell cultures. Each small colony was seen to consist entirely of RlDx or sister-pair, rather than being a mixed colony resulting from one batch of embryos to another, but this does not affect expression of the genes studied here (see below).

- We need to ensure that, when adjacent cells are seen in low density cultures, these are the clonal progeny of a single cell or sister-pair, rather than being a mixed colony resulting from two or more original cells that happened to be plated next to each other and could therefore interact. To test this we mixed unlabelled and rhodamine-labelled (RlDx) blastula cells in a ratio of 3:1, respectively, and made low density cell cultures. Each small colony was seen to consist entirely of RlDx or unlabelled cells (not shown), and was therefore formed by division of one original cell or cell-pair (Fig. 1A, inset).
Quantitative analysis of gene expression by RNase protection

We first made a quantitative analysis of gene expression in low density populations of cells attached to fibronectin after treatment with different concentrations of activin. Cells were cultured for 3–4 hours to the equivalent of stage 10.5, and were analyzed for gene expression by RNase protection. Fig. 2 shows that Xbra (Smith et al., 1991) is activated at a low concentration of activin, and reaches maximum expression at 0.45% activin. Xeomes (Ryan et al., 1996) and Xgsc (Cho et al., 1991), which has a low level of maternal mRNA, are both activated at a threefold higher concentration of activin, and continue to show progressively stronger expression up to 4% activin. Fig. 3A,B compares graphically the activin dose-response in low density (fibronectin) and reaggregated (agarose) cultures. It can be seen that the activin concentration response of cells cultured under these two conditions is indistinguishable. A similar result (not shown) was obtained with cells dispersed at low density on an agarose substrate, to which the cells did not attach. Clearly RNase protection analysis shows no effect of cell density or the degree of cell contact on the concentrations of activin at which Xbra and Xgsc are activated. We conclude that cells can sense small differences in activin concentration, and can activate different genes in a concentration-related way, in the absence of cell communication.

These results have the benefit of accurate quantitation by RNase protection, but they apply to populations of cells and do not tell us about the behaviour of individual cells.

In situ analysis of single cells

The RNase protection results just described are equally consistent with two quite different interpretations. One is that each cell senses and responds to a particular activin concentration in the same way as every other cell in the same preparation. Thus at a low activin concentration each cell will express Xbra but not Xgsc or Xeomes; at a higher concentration, each cell will express Xeomes strongly, and Xbra more weakly than at the low concentration. This would show that each cell can assess its position in a concentration gradient independently of its neighbours. The other possibility is that, while the cell population as a whole shows the dose-response described, individual cells make very different responses to the same activin concentration. It is very important to distinguish these possibilities, if we are to understand the mechanism of cell response to a morphogen.

Dissociated cells were treated with different concentrations of activin as usual, and plated onto fibronectin slides at low density and therefore in the absence of cell communication. Fig. 4 shows representative cells in situ-hybridized to probes for Xbra, Apod, Eomes or Chordin. It can be seen that the intensity of the colour reaction corresponds well with the concentration response observed in the RNase protection analyses above (Fig. 2); Xbra expression is strongest at a 0.45% dose of activin, decreasing at higher concentrations, whereas Xeomes expression increases progressively from being very weak at 0.45%, to strong expression at 4% and above. Xgsc (not shown) follows broadly the same pattern as Xeomes, but its maternal content of mRNA reduces the clarity of the effect at low activin concentrations. We have carried out a similar series of in situ hybridizations with Apod, a gene expressed at stage 10.5 (Stennard et al., 1996) as a zygotic isoform of VegT (Stennard et al., 1999). VegT (Zhang and King, 1996) is a gene also known as Xombi (Lustig et al., 1996), and Brat (Horb and Thomsen, 1997; see Stennard et al., 1997, for a review). Apod behaves like Xbra in dispersed cell preparations, in that it responds strongly to low concentrations of activin; however it does not decrease to the same extent as Xbra at higher activin concentrations (Fig. 4). We have also carried out in situ hybridization on dispersed cells using Xchordin (Sasai et al., 1994) as a probe. Xchordin shows a response pattern very similar to Xeomes (Fig. 4). The results shown in Fig. 4 were all obtained in the same experimental series. Altogether seven series with embryos from different batches of eggs were hybridized with two or more of the same probes, and gave comparable results.

We now ask how uniformly cells respond to activin concentration; are the single cells shown in Fig. 4 representative of other cells in the same samples? Low power views of the in situ hybridization preparations show apparent uniformity of expression among cells that received the same activin concentration (Fig. 5). However, the low power views seen in this figure do not reveal the detailed differences in staining intensity clearly visible in Fig. 4. Therefore to quantitate more precisely the variation within a cell population, we measured the density of stain in single cells in each low density culture. Using the procedure described in Materials and Methods, we determined the density of in situ hybridization staining for 40 cells in each sample. The results seen in Fig. 6 show substantial uniformity of gene expression among cells that received a particular concentration of activin. The validity of our method of quantitatively assessing the staining intensity in each cell is indicated by the agreement between assessment of gene expression by RNase protection (Fig. 2) and in situ hybridization (Fig. 6). As a further check on this procedure, we have used a probe for the ubiquitously expressed gene product EF1α, commonly used as a quantitative control in RNase protection experiments. The slight decline in EF1α expression at increasing concentrations of activin, as seen in Fig. 6, is expected in view of the increased cell spreading that results from activin treatment.

In conclusion, assays of gene expression at the single cell level show the same concentration-related responses as reaggregated cells. This agrees with our RNase protection results, and, most importantly, it establishes that each cell can determine its position in a concentration gradient without reference to its neighbours.

Other early genes undergo cell contact-independent activation in response to activin

In addition to the genes tested above, a number of other mesodermal and endodermal genes are expressed after activin treatment of animal cap cells. To assess the generality of our conclusions, we have tested activin responsiveness in a number of other early Xenopus genes under isolated, as compared to reaggregated, cell culture conditions. First, the magnitude of activin response, and the ability of cells to express these genes after cell dissociation, was tested using RNase protection (1) in animal caps taken from embryos which had been injected with activin mRNA (10 or 35 pg/2 cell), (2) in animal caps which had been implanted with activin protein-coated beads, or (3) in animal caps which had been dispersed, treated with activin protein (4.0% or 90 pM), and then reaggregated for culture. The transcriptional
activation of *Chordin, Cerberus* (Bouwmeester et al., 1996), *Xlim1* (Taira et al., 1992) and *Mix1* (Rosa, 1989) was detected in these animal caps, while none of these genes was expressed in caps that had no exposure to activin. All these genes are therefore activin-responsive and, for most genes, their levels of expression are similar in all of the above treatments (not shown). The response to activin by *Hex* (Newman et al., 1997) and to *Sox17b* (Hudson et al., 1997) was too low in all three samples to test the importance of cell interaction for response to activin.

For those genes that were strongly induced by activin treatment when reaggregated after dissociation (namely *Chordin, Cerberus, Xlim1* and *Mix1*), the magnitude of response was then compared in low density and reaggregated animal cap cells, to determine whether cell communication is required for gene activation following exposure to activin. Stage 8.5 animal caps were disaggregated, treated with 4% activin protein, and then cultured to stage 11 either as isolated low density cells or as reaggregates. They were then frozen for analysis by RNase protection. Whole embryos were used as positive controls, and animal caps which had no activin treatment as negative controls. The results were quantitated and a ratio calculated for each gene to show the level of expression in reaggregated cells compared to low density

### Low density cells

<table>
<thead>
<tr>
<th>Activin concentration (%)</th>
<th>Xgsc</th>
<th>Xeomes</th>
<th>Xbra</th>
<th>FGFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>WE 0</td>
<td>0.005</td>
<td>0.007</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>0.02</td>
<td>0.05</td>
<td>0.05</td>
<td>0.15</td>
<td>0.45</td>
</tr>
<tr>
<td>0.05</td>
<td>0.45</td>
<td>1.35</td>
<td>4.12</td>
<td>-</td>
</tr>
</tbody>
</table>

Fig. 2. RNase protection analysis of gene expression under low density culture conditions. *Xbra* is activated maximally at 0.45% activin. *Xgsc* and *Xeomes* reach maximum activation at 1.35% activin. WE, whole embryo.
cells. As shown in Table 1, *Chordin*, *Cerberus*, *Xlim1* and *Mix1* show a difference of less than twofold in response to activin in reaggregated versus low density cells. The same is true for *Xgsc*, included in the analysis as its activation in mesoderm cells had already been demonstrated to be largely independent of cell contact (Lemaire and Gurdon, 1994).

These results were confirmed at the single cell level using in situ hybridization to test whether isolated cells respond uniformly to activin. Dissociated animal cap cells were treated with 4% activin protein and then cultured in low density configuration on fibronectin-coated slides. We find that *Xgsc*, *Xcerberus*, *Xlim1* and *Xmix1* show uniform expression at the single cell in situ level (not shown); results with *Xbra*, *Xapod*, *Xeomes* and *Xchordin* have already been shown in Fig. 5.

**Fig. 3.** Graphic representation of gene activation response to activin concentration, for (A) low density and (B) reaggregated cell cultures. The values shown were obtained by phosphorimager assessment of gel analyses by RNase protection. B is taken from Dyson and Gurdon (1998, with permission), since the results shown in A and B are from the same experimental series. Comparable results were obtained in two other experimental series.

**Fig. 4.** Single cell analysis of gene activation in low density cell cultures. Low density preparations of cells attached to a fibronectin substrate (as in Fig. 1C) were in situ-hybridized to the probes stated (*Xbra*, *Xapod*, *Xeomes* and *Xchordin*). The colour reaction is BM purple. Each figure shows a representative clonal colony of 1-4 cells, fixed at stage 10.5. The + or – values under each figure are based on densitometry readings (see Materials and Methods). –, background value; ±, density value 2; +, ++, ++++, density values 3, 4 and 5 or more respectively (see Fig. 6). Altogether seven independent experimental series gave results similar to these (see text).
We conclude that these early genes, whether normally expressed in the mesoderm (Xbra, Xgsc, Xeomes, Xapod, Xcerberus, Xlim1, Xchordin) or endoderm (Xmix1) show a strong transcriptional response to activin, without the need for cell interactions. We cannot exclude a small effect of cell communication on the magnitude of the response, but we find that this affects gene expression by less than a factor of two (Table 1). Therefore our results with these other genes are in accord with those for Xeomes, Xbra, Xapod and Xgsc, namely that cells can respond to morphogen in the absence of cell communication.

DISCUSSION

Comparison with previous work on Xenopus
The conclusions we reach in this work clearly differ from those of the only comparable previous work (Green et al., 1994; Wilson and Melton, 1994; Symes et al., 1994). It should first be said that the main theme of the papers cited was to compare gene expression at stage 10.5 with that at stage 17. In contrast, the aim of our experiments reported here is to determine whether or not there are real differences between genes in their early (stage 10.5) dose-response to activin, and whether their response depends on cell contact and communication. The experiments described here cannot therefore be compared directly with those of the previous authors. For this reason none of our RNase protection results are inconsistent with those described by others. We should point out that Symes et al. (1994) interpret their RNase protection assays to show a broad overlapping dose response for Xbra and Xgsc, a conclusion not in accord with our results. However, their relevant figure (their Fig. 4, upper gel) shows the strongest Xbra expression at an activin concentration 4-8 times lower than their highest level.

Fig. 5. Low-power views of in situ hybridization to low density cultures on fibronectin slides. There is a uniformity of gene expression among cells at each activin concentration, although these low magnification views do not reveal the more precise differences in staining intensity seen in Fig. 4, and quantitated in Fig. 6.
of *Xgsc* expression. We do not therefore consider their results to be inconsistent with ours.

The most important contribution of our work has been to assay gene expression at the single cell level (by in situ hybridization) on isolated (low density) cultured embryonic cells. We are not aware of work in which this has been done before, possibly because it is necessary to ensure that the procedures used to permeabilize whole cells sufficiently for probe penetration do not cause detachment of cells from their substrate. We cannot therefore compare our results of this kind to previous work since that has not assessed gene expression in isolated cells. We believe that it is only by inclusion of this kind of analysis that a true understanding of cell response to morphogen concentration can be obtained.

We should comment on an apparent technical difference between our experimental procedures and those of the authors cited above. We believe that *Xenopus* embryo cells are particularly sensitive to the lack of calcium and magnesium in the medium when cultured beyond stage 10, and that this is particularly important, not surprisingly, when cells are cultured in a low-density configuration. In all our experiments, we provide calcium at or slightly above the 2 mM level characteristic of most amphibian culture media. It appears that, in the work of Green et al. (1994) and Wilson and Melton (1994), dissociated cells were cultured continuously in calcium and magnesium-free medium up till the stage of analysis. This factor may contribute to some of the differences between our results and theirs. In other work involving single cell culture but addressing different questions, calcium and magnesium were present in the culture medium (e.g. Godsave and Slack, 1991).

The results obtained here for the response of animal cap cells to activin may be compared with those described previously using equatorial cells not treated with activin (Lemaire and Gurdon, 1994). Dorsal equatorial cells, whether dissociated or in whole embryos, express the mesodermal gene *Xgsc* (Cho et al., 1991), as do activin-treated animal cap cells. *Xgsc* has a promoter with two elements that respond to two different signalling pathways (Watabe et al., 1995). The proximal element responds to the Wnt/β-catenin pathway. The distal element is required for *Xgsc* to respond to activin-class signals, which are likely to originate from a distant source such as the Nieuwkoop Centre, and therefore to involve communication between cells for establishing a concentration gradient. In the experiments described here, we provide dissociated cells with different concentrations of activin, thereby bypassing a normal cell communication stage. Once cells have received an activin signal, we now find that they do not need to communicate with each other to make an appropriate concentration-related response such as *Xgsc*. Our results are therefore consistent with previous results using dissociated *Xenopus* cells.

Finally it will be helpful to relate our results obtained here to the community effect described previously. The community effect applies to *Xenopus* embryonic mesoderm cells, but is operative only from a later stage, the mid-gastrula, onwards (Gurdon et al., 1993). In support of this point, Symes et al. (1998) observed that cells cultured at low density in Ca2+-deficient medium, and therefore unable to communicate with each other, will not activate muscle genes. Likewise, Green et al. (1994) and Wilson and Melton (1994) suggested that a community effect may help sharpen response to activin concentrations. Thus a complete response to activin concentration may have two phases. First, cells respond...
individually to activin concentrations during blastula stages. Then there is a secondary response during gastrulation, when the community effect may sharpen borders between cells expressing different genes.

**Comparison with non-amphibian work**

In *Drosophila* wing discs it has often been useful to generate clones of cells that are mutant in respect of signalling molecules or their receptors. In some cases, the mutant clones are very small, consisting of less than 10 cells. For example, the *Drosophila* mutation Saxophone encodes a defective type I receptor for DPP. In the wing disc small clones of cells lacking *sax* show the phenotype expected of their own genetic deficiency, and not that appropriate to their position in the DPP gradient as indicated by their surrounding wild-type cells (Singer et al., 1997). Therefore these cells determine their position in a concentration gradient according to their own genetic contribution, and do not depend on communication with their wild-type neighbours (Gurdon et al., 1998).

**Future directions**

Leading on from the results reported, future work will ask what later genes are expressed in isolated cells in the absence of cell communication. Our previous experiments (e.g. Gurdon et al., 1993; Carnac and Gurdon, 1997) have argued that muscle genes are not activated in the absence of cell contact and that their activation depends on a community effect. Our results presented here show that the earliest gene responses to activin do not depend on a community effect. In future work, we will want to know which other later (cyclocheximide-sensitive) genes require cell communication for a dose-dependent response to activin. Most significantly, we have now established conditions that lead to a uniform gene response to different activin concentrations, and we can ask whether all cells that make an early concentration-related response (e.g. Xbra or Xeomes) to activin behave uniformly in respect of later gene activations, or whether more complex cell interactions are involved.

We thank the Cancer Research Campaign for a programme grant to J. B. Gurdon. This work was also supported by funds from the Medical Research Council (S. D.), the European Community (T. L.), a Royal Society Howard Florey Fellowship (F. S.) and the Japanese Society for the Promotion of Science (K. S.). We thank the following for probes: J. C. Smith (Xbra); E. De Robertis (Xgsc, Chordin, Cerberus); I. B. Dawid (Xlim); P. Krieg (Hex); F. Rosa (Mix1).

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