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Synergistic action in P19 pluripotential cells of retinoic acid and Wnt3a on *Cdx1* enhancer elements

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ABSTRACT ***Cdx1*** **encodes a homeodomain protein that regulates expression of some Hox genes.** ***Cdx1*** **itself is known to be regulated in the primitive streak/tailbud by both retinoic acid (RA) and Wnt3a.** ***Cdx1*** **in eutherian mammals has two retinoic acid response elements (RAREs), located upstream and in the first intron, and each is adjacent to structural Lef/Tcf motifs. Upstream Lef/Tcf motifs respond to canonical Wnt signalling to activate** ***Cdx1*** **synergistically with RA. By combined use of reporter assays, immunofluorescence and flow cytometry in mouse P19 embryonal carcinoma cells we show that the** ***Cdx1*** **intron Lef/Tcf motif also responds to Wnt3a signalling. Synergy between individual** ***Cdx1*** **RARE and Lef/Tcf motifs can occur whether they are adjacent or distant in the gene. Part, though not all, of the** ***Cdx1*** **stimulation by RA (in absence of added Wnt3a) likely depends upon Wnt protein produced by the cells themselves, since it is inhibited by mutation of Lef/Tcf motifs, or by IWP-2, an inhibitor of Wnt production. RA and Wnt3a stimulate** ***Cdx1*** **by increasing both the proportion of P19 cells that are expressing and also their mean level of expression. The expressing/non-expressing sub-populations do not simply correspond with those that express a marker of pluripotentiality, Nanog. We conclude that RA and Wnt3a activate** ***Cdx1*** **synergistically by overlapping use of both upstream and intron enhancers, and that mouse embryonal carcinoma cell populations display heterogeneity in their response to these activators.**

KEY WORDS: *mouse Cdx1, chicken Cdx1, Lef/Tcf, retinoic acid response element, stochastic*

Introduction

The three vertebrate homeobox genes *Cdx1*, *2* and *4* are best characterized as regulators of Hox gene expression. Cdx knockout (Mallo *et al*., 2009, van den Akker *et al*., 2002) or over-expression (Gaunt *et al*., 2008) in mice produces homeotic mutations in vertebrae. The regulation of several mouse Hox genes is known to depend upon Cdx binding motifs in their *cis*-regulatory elements (Charite *et al*., 1998, Gaunt *et al*., 2004). During gastrulation and axial extension, all Cdx genes are expressed in posterior parts of the embryo. Expression here is best understood for *Cdx1* which is known to be stimulated by retinoic acid (RA) and Wnt3a signalling (Houle *et al*., 2000, Prinos *et al*., 2001). Mice knocked out for *Wnt3a* show reduced expression of *Cdx1* in the tailbud (Ikeya and Takada, 2001). Embryos over-exposed to RA show similar defects to those found in *Cdx1* over-expresser mice (Gaunt *et al*., 2008, Kessel and Gruss, 1991). *Cdx1* is also activated by Wnt activity in the gut (Lickert *et al*., 2000), where it plays a functional role (Hryniuk *et al*., 2012).

Mouse *Cdx1* is activated by RA via two retinoic acid response elements (RAREs), one upstream (Houle *et al*., 2000, Houle *et al*., 2003), and one in the first intron (Gaunt *et al*., 2003, Gaunt and Paul, 2011). It has been suggested that these RAREs may have played a part in morphological evolution since their origins coincide with divisions between major vertebrate groups (Gaunt and Paul, 2011, Gaunt and Paul, 2012). Fish and amphibian *Cdx1* genes have neither of these RAREs, whereas bird and marsupial *Cdx1* genes have only the intron RARE (Gaunt and Paul, 2011).

Each of the two RAREs in mouse *Cdx1* is adjacent to proven (upstream) or potential (intron) Lef/Tcf binding motifs. Characteristically, such motifs are of sequence [A/T][A/T][C][A][A][A/T][G] (Gustavson *et al*., 2004, Koopman, 2010) and may lie in either

Abbreviations used in this paper: EC cell, embryonal carcinoma cell; ES cell, embryo stem cell; IWP, inhibitor of Wnt production; IWR, inhibitor of Wnt response; Lef, lymphoid enhancer-binding factor; RA, retinoic acid; RARE retinoic acid response element; RAR retinoic acid receptor; Tcf, T-cell factor.

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orientation on DNA. The two motifs adjacent to the upstream RARE in mouse have been shown to enable activation of *Cdx1* by canonical Wnt (Wnt/β-catenin) signalling in embryonal carcinoma (EC) cells (Prinos *et al.*, 2001) and embryo stem (ES) cells (Lickert *et al.*, 2000). The single motif adjacent to the intron RARE is also functional, as shown by the observation that its mutation greatly reduces expression of a *Cdx1/lacZ* reporter in transgenic mice (Gaunt *et al.*, 2003), but it has not yet been shown to mediate canonical Wnt signalling. Chicken *Cdx1* lacks the upstream RARE but it does have a single Lef/Tcf structural motif (Gaunt *et al.*, 2003), of yet unproven function, conserved with one of the functional elements in mouse.

Reporter analysis upon upstream elements of mouse *Cdx1* in EC cells has shown that Wnt3a signalling alone can stimulate *Cdx1* by 5 to 10 fold (Prinos *et al.*, 2001). Wnt3a signalling has, in addition, a synergistic effect upon the action of RA on the upstream RARE of *Cdx1*. The mechanism of this is unclear but the synergy is detected both in EC cell assays (Prinos *et al.*, 2001) and *in vivo* (Pilon *et al.*, 2007).

Using P19 EC cells (McBurney, 1993) transfected with either mouse or chicken *Cdx1/lacZ* reporters, we now show that RA and Wnt3a activate *Cdx1* synergistically by overlapping use of both upstream and intron enhancers. EC cell populations display heterogeneity in their response to these activators. This heterogeneity, which appears to be a characteristic feature of pluripotential stem cell cultures, is characterized both by staining of cells in monolayer and by flow cytometry.

Results

Wnt3a is synergistic with RA at a dose where it exerts little effect alone

Fig. 1A shows the mouse and chicken *Cdx1/lacZ* reporters, and Fig. 1B shows dose response to human Wnt3a on the mouse *Cdx1/lacZ* reporter transiently transfected into P19 cells; all cells are also exposed to a fixed concentration of RA (2.5μM). A dose of 125 ng/ml Wnt3a was chosen for all subsequent experiments.

Figs. 1 C,D show absolute luciferase activity readings for mouse and chicken constructs, respectively. The activities for cultures exposed to Wnt3a alone and those exposed to RA alone are, when summed together, much less than the activities for cultures exposed to both Wnt3a and RA. This is in spite of the fact that the summed values represent readings for twice the number of cells. We therefore conclude that Wnt3a and RA act synergistically in their effect upon Cdx1 activity. In these experiments, Wnt3a plus RA stimulates luciferase activity 3.1 fold (mouse; Fig. 1C) and 2.9 fold (chicken; Fig. 1D) relative to RA alone. From the data of Fig 1 C,D, most (about 99%) of this can be attributed to synergistic action between Wnt3a and RA since Wnt3a alone at this dose exerts little stimulatory effect relative to cultures exposed to no Wnt3a. In some experiments that follow (Fig. 2A, 2C, 3A, 3C) we present Wnt3a plus RA results as fold change relative to RA alone, and we regard this as mainly due to synergy between these compounds.

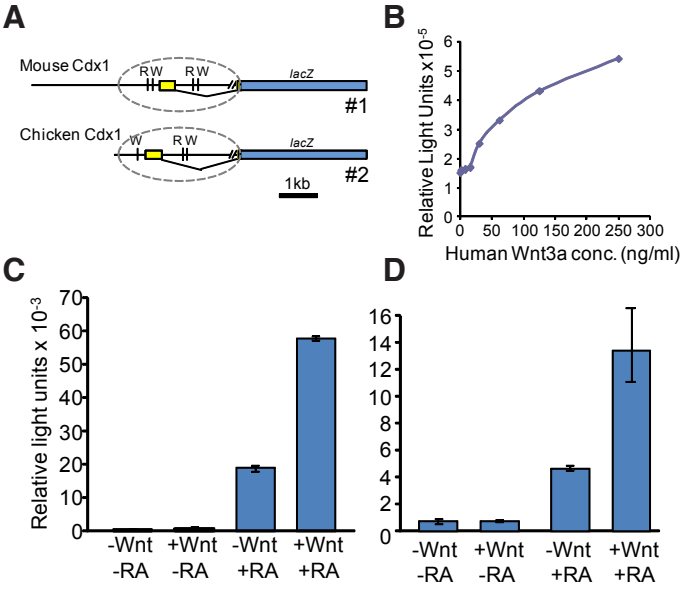


Fig 1. Wnt3a exerts a synergistic effect upon retinoic acid stimulation of β-galactosidase expression from *Cdx1/lacZ* constructs transiently transfected into P19 cells. (A) *Mouse (#1) and chicken (#2) constructs showing the position of upstream and intron retinoic acid (RA) response elements (R) and Lef/Tcf motifs (W). Upstream W in mouse represents a pair of adjacent, functional Lef/Tcf motifs (Lickert* et al., *2000), whereas upstream W in chicken, and intron W in mouse and chicken, each represent a single Lef/Tcf motif (Gaunt* et al., *2003).* **(B)** *Dose response curve for human Wnt3a on mouse* Cdx1/lacZ *in P19 cells. Each cell monolayer received RA (2.5μM) plus one of the indicated doses of Wnt3a for 18 hours prior to β-galactosidase assay.* **(C)** *Mouse* Cdx1/lacZ *(#1).* **(D)** *Chicken* Cdx1/lacZ *(#2). In C and D, the cell culture data within each bar chart are from a single experiment, and each bar shows average values for three replicate cultures given 18 hours incubation in activators (Wnt3a, 125ng/ml; RA, 2.5μM; Wnt3a plus RA; or none). Range bars are shown.*

Both intron and upstream Lef/Tcf motifs respond to Wnt3a to act synergistically with RA, and the RARE and Lef/Tcf motifs may or may not be adjacent

First, we examined mouse *Cdx1* constructs (#3 to #6) in which the intron RARE is intact but the upstream RARE is mutated. In Fig. 2A, all cultures are exposed to RA. Response is stimulated about 3.9 fold by Wnt3a when all Lef/Tcf sites are intact (#3) but is reduced by mutation of either upstream (#4) or intron (#5) Lef/Tcf sites. Response to Wnt3a is further reduced by mutation of all indicated Lef/Tcf motifs (#6). The requirement for intact Lef/Tcf motifs provides evidence that both upstream and intron sites are responding by the canonical Wnt (or Wnt/β-catenin) pathway. This supports a conclusion published earlier for the upstream Lef/Tcf sites (Prinos *et al.*, 2001).

Next, we examined constructs #7 to #10 in which the upstream RARE is intact but the intron RARE is mutated. In Fig. 2C, response to RA is stimulated about 3.7 fold when all Lef/Tcf sites are intact (#7), but is reduced by mutation of either intron (#8) or upstream (#9) Lef/Tcf sites, and is further reduced by mutation of all sites (#10).

These data show (i) that both upstream and intron Lef/Tcf sites are able to respond to Wnt3a in its synergistc effect upon RA activation of *Cdx1*, and (ii) that intron and upstream RAREs can each co-operate with Lef/Tcf sites located both adjacently (#4 and #8) and at a distance (#5 and #9).

Chicken *Cdx1* has only the intron RARE. As for mouse, Wnt3a synergistic activation of the RA response (Fig. 3A, #2) was inhibited by mutation in either the intron (#11) or upstream (#12) Lef/Tcf sites, and in chicken, though not always in mouse, Wnt3a

synergistic action on RA was completely nullified by mutation in both (#13). A possible explanation for this observation could be existence of additional mouse Lef/Tcf motifs not mutated in our study. For example, two additional upstream motifs were earlier identified in mouse *Cdx1* (Lickert *et al.*, 2000) although these did not appear highly functional. In chicken, no additional Lef/Tcf motifs are present within the basic *Cdx1/lacZ* construct.

Mutation of Lef/Tcf sites reduces the response to RA alone, but does not abolish it

For both mouse (Fig. 2B, 2D) and chicken (Fig. 3B) constructs mutation of either upstream or intron Lef/Tcf sites reduces the response to RA alone (no added Wnt3a) but mutation of all identified Wnt3a sites does not abolish all response to RA. This result suggests that (i) part of the *Cdx1* response to RA alone may be amplified by endogenous Wnt/β-catenin activity in the P19 cells, but (ii) some response to RA is likely to persist in absence of any such endogenous activity. We examine these proposals later by flow cytometry.

In the converse experiment, mutation of the chicken RARE produces little reduction in the effect of Wnt3a alone (Fig. 3D). This indicates that the slight stimulation of *Cdx1* by Wnt3a can occur in the absence of any RA signalling.

Synergy between RA and Wnt3a occurs at the level of the Cdx1 gene

Previous studies on the upstream regulatory region of *Cdx1* showed that synergy is inhibited by mutation in either the RARE or the Lef/Tcf sites (Prinos *et al.*, 2001). That is, RA synergizes with Wnt3a at the level of the *Cdx1* promoter. By use of chicken *Cdx1/lacZ* constructs we examined whether the same is true for the intron regulatory elements. In the presence of both RA and Wnt3a, synergy is inhibited by mutation in either the intron Lef/Tcf motif (cf #12 and #13; Fig. 3A) or the intron RARE (cf #12 and #14; Fig. 3C). This suggests that synergy requires binding to both RARE and Lef/Tcf motifs, and tends to exclude mechanisms proposed in other cell systems, such as Wnt3a up-regulation of RAR-γ (Szeto *et al.*, 2001) or RA up-regulation of β-catenin (Liu *et al.*, 2002).

RA and Wnt3a/RA stimulation of Cdx1 change both the proportion of expressing P19 cells and their mean levels of expression

We prepared a cell clone stably transfected with mouse *Cdx1/lacZ* construct (P19Cdx1/lacZ), and examined for expression at the single cell level using anti-β-galactosidase antibody (Fig. 4A). The proportion of labelled cells increased upon addition of RA (Fig. 4B), and this proportion increased further upon addition of both RA and Wnt3a (Fig. 4C). Apparently similar proportions of labelled and unlabelled cells were seen in subclones picked as single cells by micropipetting from the original clone (not shown).

Next, we prepared a cell clone (P19Cdx1/GFP) stably transfected with a mouse *Cdx1/GFP* construct (Fig. 5G), which contains the same *Cdx1* sequence as the *Cdx1/lacZ* construct. In the flow cytometry experiment shown in Fig. 5, P19Cdx1/GFP cells showed labelling frequencies of 0.4% without RA or Wnt3a (Fig. 5B), 1.6% with Wnt3a alone (Fig. 5C), 15.7% with RA alone (Fig. 5D) and 36.7% with RA plus Wnt3a (Fig. 5E). We also examined the effect of an Inhibitor of Wnt Response, IWR-1, which is known to inhibit the effect of Wnt signalling by promoting degradation of β-catenin (Chen *et al.*, 2009). IWR-1 reduced the proportion of cells responding to RA to 7.6% (Fig. 5F). This suggests again that part of the response to RA alone is due to synergy with Wnt/β-catenin signalling. Fig. 5 also shows the mean (geometric mean) fluorescence intensities of the labelled populations. It is seen that fluorescence intensity

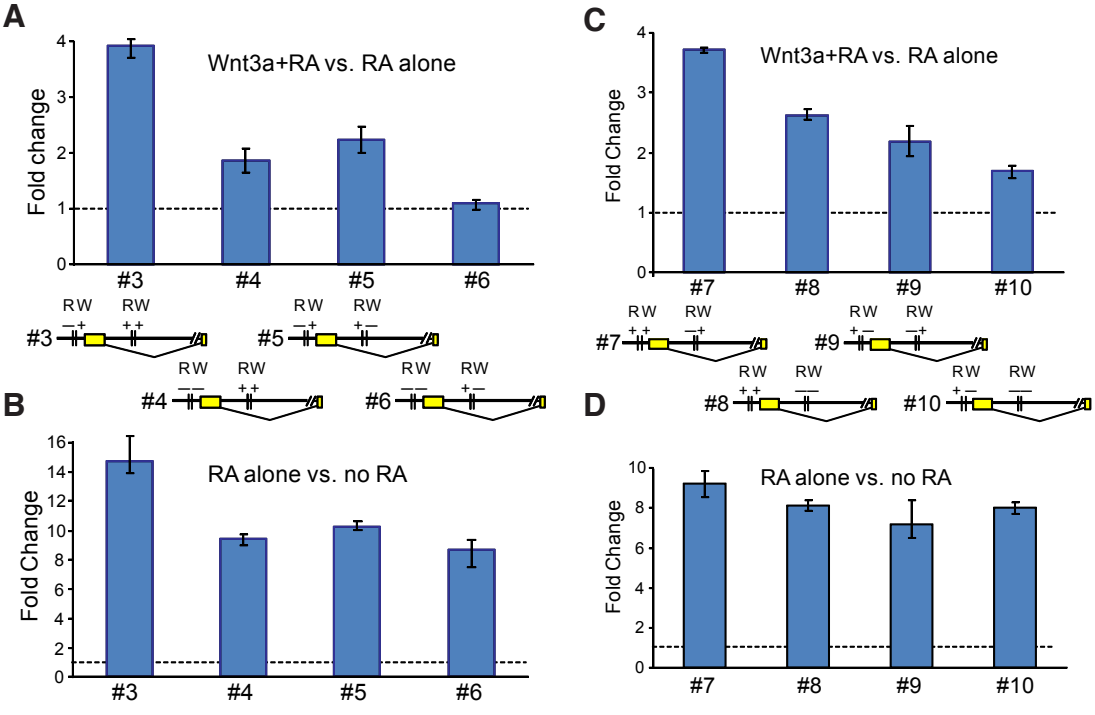


Fig 2. Effect of mutations in Lef/Tcf and retinoic acid response motifs upon β-galactosidase expression of mouse *Cdx1/lacZ* reporters transiently transfected into P19 cells. (A,B) *constructs in which only the intron retinoic acid response element (RARE) motif is intact;* **(C,D)** *constructs in which only the upstream RARE motif is intact. Cartoons represent the area of construct #1 encircled in Fig 1A, and show RARE (R) and Lef/Tcf (W) sites as intact (+) or mutated (-). In (A,C) fold change is the effect of Wnt3a plus RA relative to RA alone (latter shown as dotted baseline). In (B,D) fold change is the effect of RA alone relative to no RA (latter shown as dotted baseline). The cell culture data within each bar chart are from a single experiment, and each bar shows average values for three replicate cultures given 18 hours incubation in activator(s) relative to the average of three replicates not given the activator, as specified. Range bars and activator concentrations as for Fig 1.*

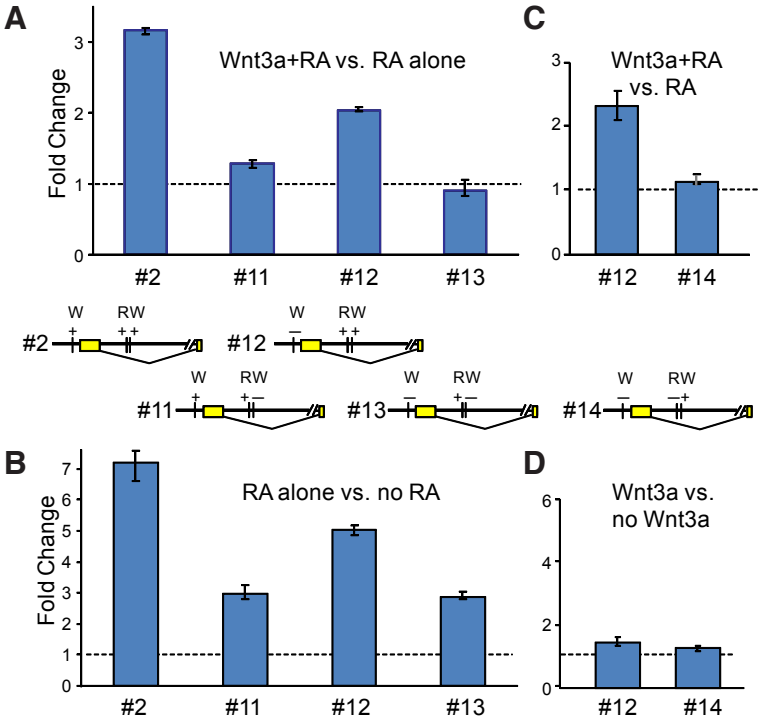


Fig 3. Effect of mutations in Lef/Tcf and retinoic acid response motifs upon β-galactosidase expression of chicken *Cdx1/lacZ* reporters transiently transfected into P19 cells. *Cartoons represent the area of construct #2 encircled in Fig 1A, and show RARE (R) and Lef/Tcf (W) sites as intact (+) or mutated (-). In* **(A,C)** *fold change is the effect of Wnt3a plus RA relative to RA alone (latter shown as dotted baseline). In* **(B),** *fold change is the effect of RA alone relative to no RA (latter shown as dotted baseline). In* **(D)**, *fold change is the effect of Wnt3a alone relative to no Wnt3a (latter shown as dotted baseline). Cell culture, activator concentrations, graphs and range bars as for Fig. 2.*

increases in line with increase in the proportion of labelled cells, and decreases upon addition of IWR-1.

It is clear that all cells in p19 cultures do not respond in an identical way to RA and to Wnt3a. Heterogeneity in expression within pluripotential cell cultures is recognized for a variety of differentiation markers (Canham *et al.*, 2010, Chambers *et al.*, 2007, Hayashi *et al.*, 2008, Toyooka *et al.*, 2008), and has been attributed either to stochasticity in the mode of transcriptional control (Harper *et al.*, 2011, Li and Xie, 2011, Munoz-Descalzo *et al.*, 2012) or else to co-existence of cells at varying and interconvertible stages of differentiation (Hayashi *et al.*, 2008). Loss of Nanog expression provides an early marker of differentiation from inner cell mass-like cells to epiblast-like cells, and the cells within pluripotential stem cell cultures are heterogeneous in their levels of Nanog expression (Chambers *et al.*, 2007). After addition of RA, the P19Cdx1/lacZ expressing sub-population overlapped with both Nanog high- and low-expressing cells (Fig. 4D-F). This indicates that Cdx1/lacZ expression is not simply related to the state of cell differentiation as assessed by Nanog expression.

Wnt secretion by P19 cells amplifies response to RA

We have concluded above that part of the *Cdx1* response to RA alone is amplified by endogenous Wnt/β-catenin activity in the P19 cells. To test whether this is due to Wnt protein secreted

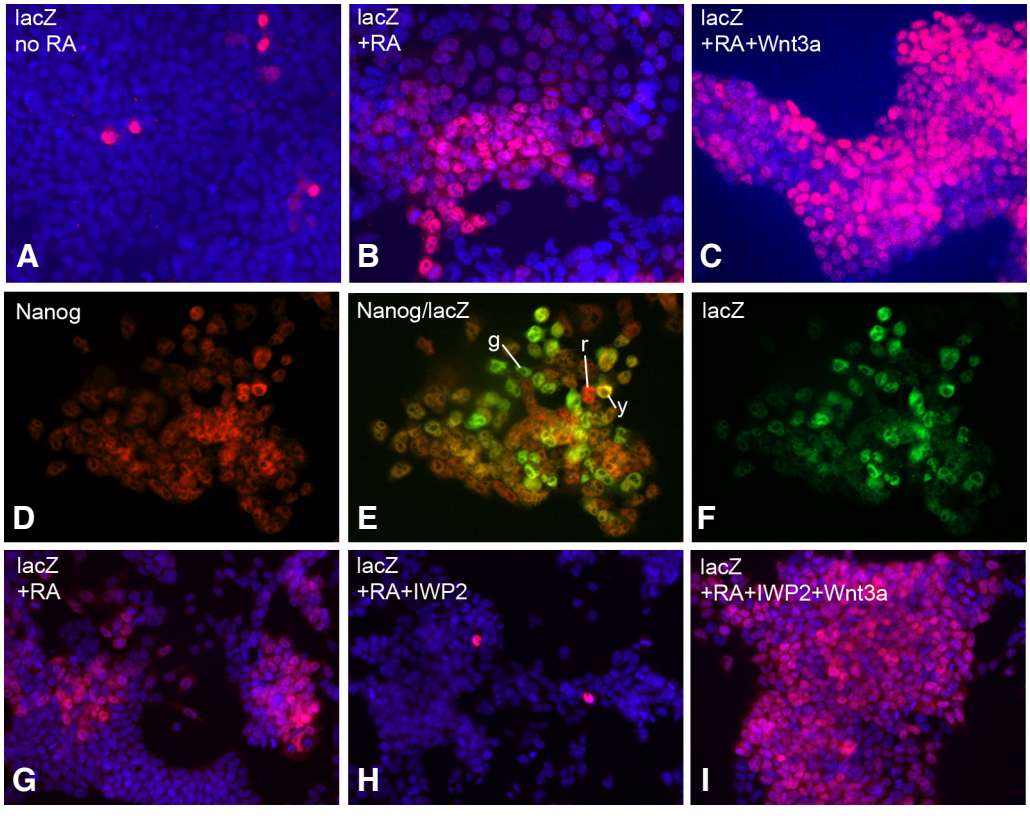


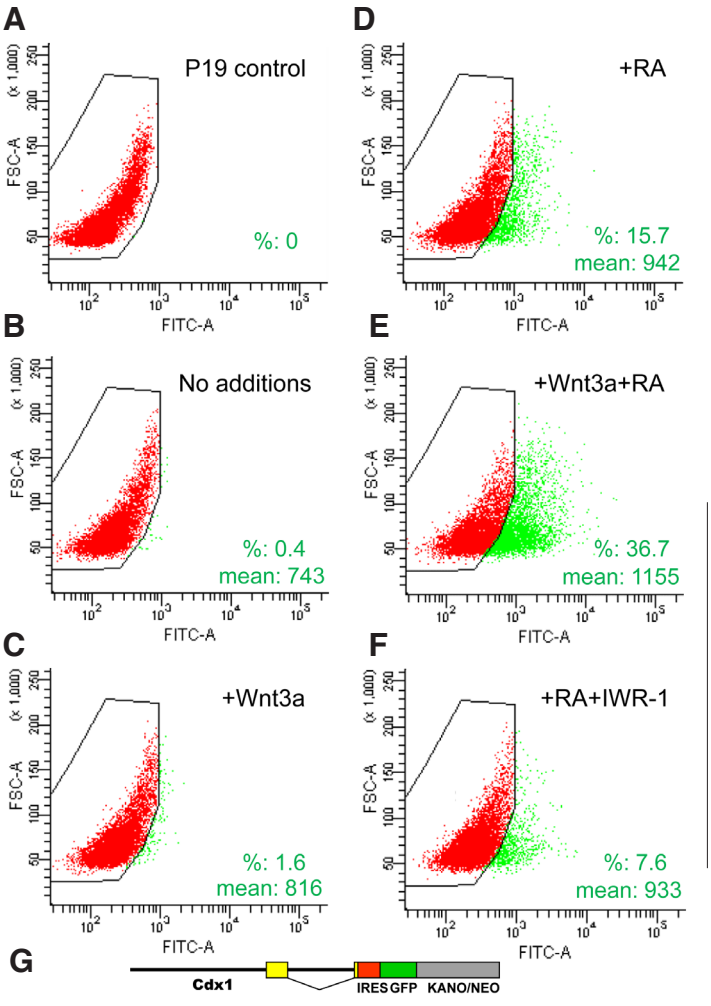
Fig 4. Retinoic acid and retinoic acid plus Wnt3a increase the proportion of Cdx1/lacZ P19 cells that express *Cdx1* transgene. (A-C, G-I) Cdx1/lacZ *transgenic cells fixed and stained to show lacZ nuclear expression (red). Cells are counterstained with DAPI (blue). Cells were either untreated* **(A)**, *treated with RA* **(B,G)**, *RA plus Wnt3a* **(C)**, *RA plus IWP-2 (H), or RA plus IWP-2 plus Wnt3a (I).* **(D-F)** *RA treated* Cdx1/lacZ *cells stained for both Nanog (red)* **(D)** *and lacZ (green)* **(F)**. *In* **(E),** *images (D) and (F) are blended, and show green (g), red (r), and yellow (y) cells. Yellow cells express high levels of both Nanog and lacZ. Each horizontal row is a separate experiment. IWP-2 was used at 2μM; activator concentrations were as for Fig 1.*

by the P19 cells we examined the effect of an Inhibitor of Wnt Production, IWP-2, which inhibits the processing of Wnt protein prior to release from cells (Chen *et al.*, 2009). In the flow cytometry experiment shown in Fig. 6, 5% of cells expressed Cdx1/GFP in RA (Fig. 6A), but only 1.1% in RA plus IWP-2 (Fig. 6B). There was also reduction in the mean fluorescence intensity of the labelled sub-population. These inhibitions were not due to non-specific toxicity since they were largely overcome by adding Wnt3a to the medium (compare Figs. 6 B,C,D).

By immunofluorescence on P19Cdx1/lacZ cells, IWP-2 inhibited the effect of RA (Fig. 4G), apparently leaving only a few heavily labelled cells (Fig. 4H) similar to cultures with no additions (Fig. 4A). Cultures which received RA, IWP-2 and Wnt3a had widespread expression by the majority of the cells (Fig. 4I). It was clear that labelled cells after RA treatment were commonly grouped together (Fig. 4 B,G). We considered the possibility that labelled groups may form around cells which are secreting high levels of Wnt protein into the culture medium. While this may be true in part it is difficult to explain why some segregation is also seen in cultures grown in presence of RA plus added Wnt3a (Fig 4 C,I). It is possible that segregated populations differ in their state of differentiation, although not with respect to Nanog expression (Fig 4 D-F).

Evolutionary origins of Cdx1 Lef/Tcf motifs

We earlier showed that the intron Lef/Tcf motif, located near to the intron RARE, is structurally conserved in anole lizard, bird, marsupial and eutherian mammal (Gaunt and Paul, 2011) (Fig. 7B). We find no Lef/Tcf motif of structure [A/T][A/T][C][A][A][A][G] in the first introns of fugu or *Tetraodon Cdx1* (two fish species; not shown). The intron RARE and intron Lef/Tcf motifs might therefore have been acquired, perhaps together (Fig. 7B), as two synergistic components within a new *cis*-regulatory element. We found the upstream RARE only in eutherian mammals (Gaunt and Paul, 2011) (Fig. 7B). However, the upstream Lef/Tcf motif(s) were clearly acquired earlier in evolution than the upstream RARE since we find a structurally conserved element upstream of the first exon in species from coelocanth to eutherian mammal (Fig. 7 A,B). We have shown that these elements are functional in both mouse and chicken (Fig. 2,3).



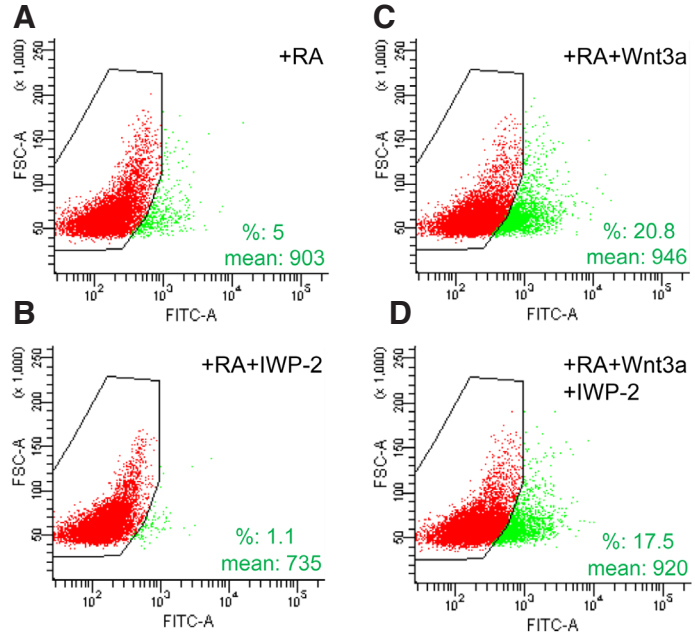


Fig 5 (Left). Flow cytometry to analyse the effect of retinoic acid and Wnt3a upon Cdx1/GFP expressing cells. (A) *Non-transgenic P19 cells with plot gated to define the limits of non-fluorescent cells (red).* **(B-F)** Cdx1/GFP *transgenic cells analysed after 18 hours exposure to no additions (B), Wnt3a (C), RA (D), Wnt3a plus RA (E), RA plus IWR-1 inhibitor (F). Cells in (F) also received pre-incubation in IWR-1 for 6 hours. Fluorescent cells are shown as green dots. Results shown are from the same experiment, and ten thousand cells were recorded in each plot. FITC-A, fluorescein isothiocyanate fluorescence intensity (arbitrary units); FSC-A, forward scatter; %: fluorescent cells (green) as percentage of total cells shown; mean: geometric mean fluorescence intensity of the labelled population.* **(G)** *Structure of the* Cdx1/GFP *transgene. IWR-1 was used at 5μM; activator concentrations were as for Fig 1.*

Fig 6 (Right). Flow cytometry to analyse the effect of an Inhibitor of Wnt Production, IWP-2, upon Cdx1/GFP expressing cells. (A-D) *Cells analysed 18 hours after exposure to RA (A), RA plus IWP-2 (B), RA plus Wnt3a (C), RA plus Wnt3a plus IWP-2 (D). Cells in (B,D) also received pre-incubation in IWP-2 for 6 hours. Experimental procedure and graph labels as for Fig 5; activator and IWP-2 concentrations as for Fig 4.*

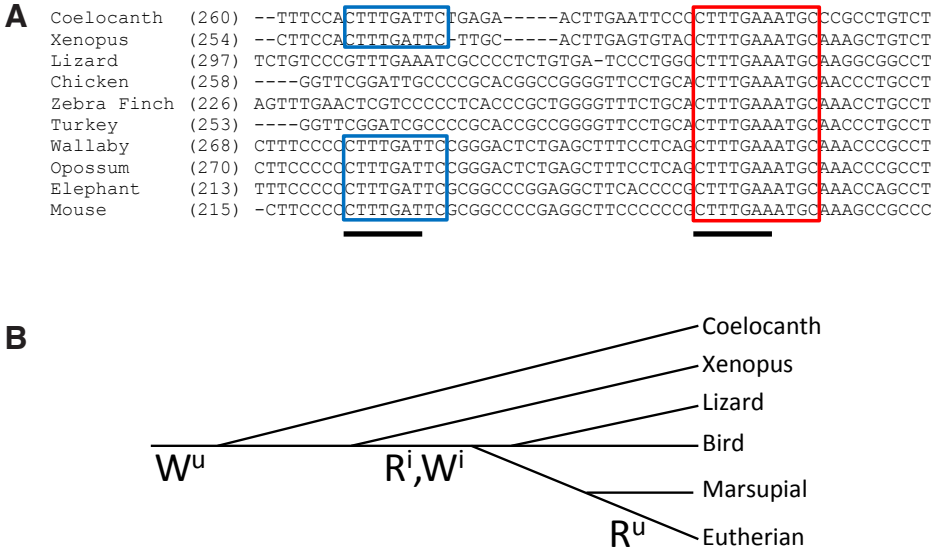


Fig 7. Cdx1 Lef/Tcf motifs and RAREs in vertebrate phylogeny. (A) *Alignment of* Cdx1 *upstream Lef/Tcf motifs.* Xenopus *is* Xenopus tropicalis*; Lizard is Anole Lizard. Numbers indicate base pairs upstream of the ATG for the start of each sequence shown. Red box, fully conserved sequence; blue, highly conserved. Underlined are the two motifs shown functional in mouse (Lickert* et al.*, 2000).* **(B)** *Suggested origins of the regulatory motifs. Additional sequence alignments supportive of this are published (Gaunt and Paul, 2011).* W^u, W^i, *upstream and intron Lef/Tcf motifs.* R^u, R^i, *upstream and intron RAREs.*

Discussion

Regulation of homeobox genes analysed in EC/ES cells

*Cdx*1 activation by Wnt3a and RA can be characterized in EC/ES cells (Houle *et al.*, 2000, Lickert *et al.*, 2000, Prinos *et al.*, 2001), and the mechanisms are apparently similar *in vivo* (Houle *et al.*, 2003, Pilon *et al.*, 2007). Other examples where homeobox gene regulations have been productively studied in mouse pluripotential cells cultured *in vitro* are RA activation of Hox genes in EC cells (Simeone *et al.*, 1991), Wnt pathway activation of *Cdx4* in EC cells (Pilon *et al.*, 2006), BMP4 and Wnt3a synergistic activation of *Cdx1* and *Cdx4* in ES cells (Lengerke *et al.*, 2008), and Cdx2 activation of *Cdx4* in EC cells (Savory *et al.*, 2011).

Synergy between Cdx1 RAREs and Lef/Tcf motifs

We show that the *Cdx1* intron Lef/Tcf motif, like the upstream motifs (Lickert *et al.*, 2000, Prinos *et al.*, 2001), is responsive to canonical Wnt3a stimulation. Wnt3a stimulation of the intron element is synergistic with RA stimulation, as has also been found for the upstream elements (Prinos *et al.*, 2001). Synergy between individual *Cdx1* RARE and Lef/Tcf motifs can occur whether they are adjacent (e.g., both intronic) or distant (one intronic and one upstream). The mechanism of synergy is unknown but for both upstream (Prinos *et al.*, 2001) and intron RAREs it apparently requires binding to both a RARE and at least one Lef/Tcf site within the *Cdx1* construct. That is, RA synergizes with Wnt3a at the level of the *Cdx1* gene. One possibility is that binding of Lef/Tcf may sensitise the adjacent RARE by an effect upon chromatin structure (Pilon *et al.*, 2007). Our new findings indicate that this must be effective over a distance of up to 1.7 kb (distance between the upstream RARE and the intron Lef/Tcf site in mouse *Cdx1*). Binding of Lef1 or Tcf1 complexes introduces a 130° bend in the DNA, and this may be important in the change in chromatin structure (Giese *et al.*, 1992, Giese *et al.*, 1995, Koopman, 2010).

We show that synergy between Wnt and RA signalling occurs even in absence of added Wnt3a. Thus, we found that we could reduce the response to RA alone by either (i) mutation of *Cdx1* Lef/Tcf sites, or (ii) addition of IWP-2, which blocks Wnt processing prior to release from cells. We conclude that P19 cells must normally release Wnt protein into their medium and that this acts synergistically with added RA. It is already reported that ES cells secrete a wide range of different Wnt proteins (Biechele *et al.*, 2011, ten Berge *et al.*, 2011).

Heterogeneity in expression within EC cell cultures

We found that response to RA and Wnt3a is due to both (i) change in the proportion of expressing cells in the P19 cultures, and (ii) change in the level of expression. The first of these indicates heterogeneity in expression within the P19 EC cell cultures. Heterogeneity in expression is already extensively described for ES cell cultures. For example, Canham *et al.,* studied Hex gene expression, a marker of primitive endoderm, and found that a Hex/YFP construct is variably expressed (Canham *et al.*, 2010). Fractions enriched by FACS for high or low expression regenerate the full range of expression after about 24 hours. Similar findings have been reported for ES cells expressing the pluripotency markers *Stella (Hayashi et al., 2008), Nanog* (Chambers *et al.*, 2007) and *Rex1* (Toyooka *et al.*, 2008).

Heterogeneity in ES/EC cell expression could be explained by two different models. In a stochastic expression model the cells need not differ in their states of differentiation, but they are considered to express a gene only with a certain probability due to the rate limiting nature of transcription factor binding to DNA (Harper *et al.*, 2011, Li and Xie, 2011, Munoz-Descalzo *et al.*, 2012). Here, RA would increase the probability that an EC cell expressed *Cdx1*, and Wnt3a would increase this further. In contrast, however, most authors consider that ES cell heterogeneity in gene expression is largely due to their containing a dynamic equilibrium of different, inter-convertible cell states, in particular inner cell mass (ICM)- like and epiblast-like (Canham *et al.*, 2010, Hayashi *et al.*, 2008, Toyooka *et al.*, 2008). These different cell states are distinguished by levels of the pluripotency marker Nanog (Chambers *et al.*, 2007, Dutta, 2013). In P19 cultures stimulated by RA we found no correlation between cells expressing Cdx1/lacZ and those expressing Nanog. However, since these transcription events may occur at non-identical positions along an ICM/epiblast transition we cannot rule out cell differentiation as a possible source of heterogeneity in Cdx1/lacZ expression.

Origins of Lef/Tcf motifs and RAREs during vertebrate evolution

We earlier presented reasons to support our hypothesis that the RAREs in *Cdx1* may have played roles in morphological evolution (Gaunt and Paul, 2011, Gaunt and Paul, 2012). First, their origins coincide with divisions between major vertebrate groups: the intron RARE at the amphibian/amniote split, and the upstream RARE at the marsupial/eutherian split (Fig. 7B), and these evolutionary transitions are accompanied by morphological changes in structures known to be regulated by *Cdx1* (Gaunt and Paul, 2011). Second, the separate evolutionary origins for *Cdx1* RAREs contrast with those for Hox gene RAREs, which are typically found to be conserved from fish to mammals (Gaunt and Paul, 2012).

The *Cdx1* intron RARE and Lef/Tcf motifs may have co-evolved at the origin of the amniotes (Fig. 7B), forming synergistic elements within a new enhancer complex, since we found no Lef/Tcf motifs of structure [A/T][A/T][C][A][A][A][G] in the first introns of *Cdx1* from two fish species. In contrast, the upstream Lef/Tcf motif(s) clearly originated earlier than the upstream RARE (Fig. 7B), and we now show that the upstream region of chicken *Cdx1*, which has no RARE (Gaunt and Paul, 2011), does possess a functional Lef/Tcf motif conserved in sequence with mouse. This structurally conserved upstream Lef/Tcf motif is present in coelacanth and also *Xenopus*, a species where *Cdx1* shows no response to RA (Shiotsugu *et al.*, 2004), indicating that this motif has a role independent of RA signalling. This role may include Wnt/β-catenin regulation of *Cdx1* expression in the intestine (Lickert *et al.*, 2000).

Conclusions

Our main conclusions are as follows. 1) The *Cdx1* intron Lef/Tcf motif, like the upstream motifs, is responsive to Wnt3a stimulation. 2) Synergy between individual *Cdx1* RARE and Lef/Tcf motifs can occur whether they are adjacent or distant. 3) For the intron elements, as earlier found for the upstream elements (Prinos *et al.*, 2001), synergy apparently requires binding of RA and Wnt3a signalling components to the DNA. 4) Part, though not all, of the *Cdx1* stimulation by RA (in absence of added Wnt3a) likely depends upon Wnt protein produced by the cells themselves. 5) RA and Wnt3a stimulate *Cdx1* in P19 cells by increasing both the proportion of cells that are expressing and their mean level of expression. The expressing/non-expressing sub-populations do not simply correspond with those expressing a marker of pluripotentiality, Nanog.

Materials and Methods

Plasmids

The mouse and chicken *Cdx1/lacZ* constructs used are shown in Fig. 1A. The mouse construct is as described before (Gaunt *et al.*, 2003, Gaunt and Paul, 2011). The chicken construct is also as before except that the upstream sequence terminates at the *Xho*1 site (Gaunt *et al.*, 2003); this is to eliminate all putative structural Lef/Tcf motifs in the upstream sequence apart from the one identified 217-223 bp upstream of the ATG (Fig. 7A).

Mutations were introduced into the mouse *Cdx1/lacZ* construct as summarized in the cartoons between upper and lower bar charts in Fig. 2. Constructs #3 to #6 have the upstream RARE mutated from GAAGGGTCGTGACCCCTAA to GAAGGGGTCGACCCCCTAA, introducing a *Sal*1 site. Constructs #7 to #10 have the intron RARE mutated from TGAACTCTTGACCC to CATATGCCGACTAG, introducing a *Nde*1 site. Constructs #4 and #9 have the two upstream Lef/Tcf motifs (underlined in Fig. 7A) replaced by *Eco*R1 and *Spe*1 sites. Constructs #5 and #8 have the intron Lef/Tcf mutated from TTCAAAG to ACGCGTG, introducing a *Mlu*1 site. Constructs #6 and #10 have both of the above Lef/Tcf mutations.

Mutations were introduced into the chicken *Cdx1/lacZ* construct as summarized in the cartoons between upper and lower bar charts in Fig. 3. Construct #11 has the intron Lef/Tcf motif mutated from TTCAAAG to ACTAGTT, introducing a *Spe*1 site. Construct #12 has the upstream Lef/Tcf motif mutated from CTTTGAA to CGAATTC, introducing a *Eco*R1 site. Construct #13 has both of the above Lef/Tcf mutations. Construct #14 resembles #12, but also has the RARE mutated from TGAACTCCTGACCC to CTCGAGCCGACTAG, introducing an *Xho*1 site.

A *Cdx1/GFP* construct (Fig. 5G) contains the same mouse *Cdx1* regions as the *Cdx1/lacZ* construct spliced upstream of *IRES* and *GFP* sequences taken from *pEGFP-N1* (Clontech). GFP, unlike lacZ, is therefore produced as a non-fusion protein.

Cell culture, transfection and β-galactosdase assays

P19 cell culture, transfections and β-galactosidase chemiluminescence assays were as described earlier (Gaunt and Paul, 2011). Each assay was performed as three biological replicates, with each graph bar (Figs. 1 to 3) showing the mean results obtained from three separate monolayers seeded at the same time with equal numbers of cells. The range of readings obtained between the three separate monolayers is shown in the range bars.

To prepare stably transformed cell lines, constructs had the *kano/neo* gene from *pEGFP-N1* inserted downstream of the marker gene (e.g. Fig. 5G), and they were linearized in the *pBluescript* polylinker prior to transfection. Post transfection, cells were plated sparsely in presence of 1600μg per ml G418 to allow cell selection. After a further three days, surviving cells were allowed to grow up as colonies in 400μg per ml G418. Expressing clones were sub-cloned by picking single cells in a micropipette and were maintained in 400μg per ml G418. For each of the two transgenic cell lines described in this paper (P19Cdx1/lacZ and P19Cdx1/GFP) similar findings were obtained for at least two independently-selected clones (not shown). *All-trans* retinoic acid (Sigma) was used at a concentration of 2.5μM. Human Wnt3a (R&D Systems) was, unless otherwise stated, used at 125 ng/ml. Wnt inhibitor IWP-2 (Calbiochem) was used at 2μM. and IWR-1 (Sigma) was used at 5μM.

Immunofluorescence and flow cytometry

For immunofluorescence microscopy, monolayers were fixed for 15 min in 4% paraformaldehyde, washed in PBS, and then permeabilized in 0.5% Triton X-100 for 5 min prior to labelling by a standard protocol (abcam). Anti β-galactosidase mouse monoclonal antibody was clone 40-1a from DSHB (University of Iowa). Anti Nanog rabbit polyclonal antibody was from abcam (cat. no. ab80892). Secondary antibodies, labelled with Alexa Fluor 488 or 568, were from Invitrogen (cat. nos. A11029, A11004, A10042). For flow cytometry, cells were trypsinized, passed through 40 micron filters and then analysed live using an LSR II (BD Biosciences). The mean intensity of fluorescence in the labelled population was calculated as its geometric mean, as is recommended for logarithmically displayed flow cytometry data (Ormerod, 2008).

Sequence analysis

Regions of conservation were identified in sequences from the ENSEMBL database. Accession numbers were as previous (Gaunt and Paul, 2011), or were: fugu, ENSTRUG00000000997; *Tetraodon*, ENSTNIG00000000119; coelocanth, ENSLACG00000014957; chicken, ENSGALG00000005679; zebra finch, ENSTGUG00000000941; mouse, ENSMUSG00000024619. Sequence alignments were made using Clustal Omega (EMBL-EBI).

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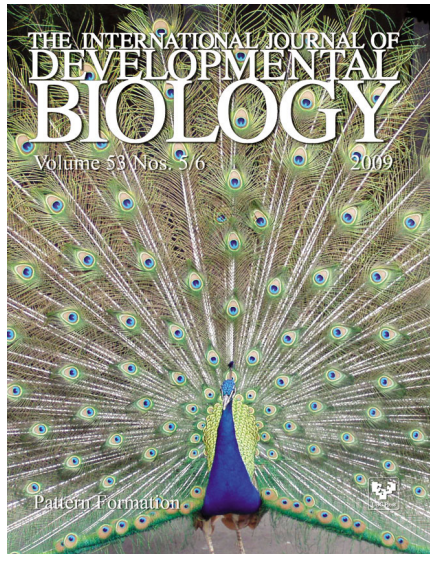

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