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The Competence of *Xenopus* Blastomeres to Produce Neural and Retinal Progeny is Repressed by Two Endo-Mesoderm Promoting Pathways

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Abstract

Only a subset of cleavage stage blastomeres in the *Xenopus* embryo is competent to contribute cells to the retina; ventral vegetal blastomeres do not form retina even when provided with neuralizing factors or transplanted to the most retinogenic position of the embryo. These results suggest that endogenous maternal factors in the vegetal region repress the ability of blastomeres to form retina. Herein we provide three lines of evidence that two vegetal-enriched maternal factors (VegT, Vg1), which are known to promote endo-mesodermal fates, negatively regulate which cells are competent to express anterior neural and retinal fates. First, both molecules can repress the ability of dorsal-animal retinogenic blastomeres to form retina, converting the lineage from neural/retinal to non-neural ectodermal and endo-mesodermal fates. Second, reducing the endogenous levels of either factor in dorsal-animal retinogenic blastomeres expands expression of neural/retinal genes and enlarges the retina. The dorsal-animal repression of neural/retinal fates by VegT and Vg1 is likely mediated by Sox17a and Derriere but not by XNr1. VegT and Vg1 likely exert their effects on neural/retinal fates through at least partially independent pathways because Notch1 can reverse the effects of VegT and Derriere but not those of Vg1 or XNr1. Third, reduction of endogenous VegT and/or Vg1 in ventral vegetal blastomeres can induce a neural fate, but only allows expression of a retinal fate when both BMP and Wnt signaling pathways are concomitantly repressed.

Keywords

VegT; Vg1; Xnr1; derriere; sox3; Notch1; rx1; endodermin; Xbra; retina specification

Introduction

The elucidation of the molecular and cellular mechanisms that control how early embryonic cells are specified to express different fates is central to our understanding of how vertebrate embryos develop into different tissues. The vertebrate retina is a particularly good model for studying fate specification because of its accessibility during developmental stages, a wealth of tissue and stage-specific markers and a number of mutations and diseases that affect its development and function. The developmental program that regulates the specification of retinal fate is comprised of multiple steps that influence the competence of embryonic cells to

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form retina (reviewed in Williams and Moody, 2004;Zaghloul et al., 2005). Although events during neural plate and optic cup formation are critical for defining the retina-specific stem cells and the many cell phenotypes that are derived from them (Marquardt, 2003;Hatakeyama and Kageyama, 2004), earlier developmental decisions have been shown to significantly impact the ability of embryonic cells to express a retinal fate in *Xenopus*, in whose embryos specific precursors of the retina can be identified at early cleavage stages.

The potential to form retina is restricted to the dorsal-animal quadrant at early cleavage stages in normal *Xenopus* embryos (Fig. 1; Huang and Moody, 1993). These cells are a subset of those that give rise to the entire nervous system (Moody, 1987a,b). Other cells in the animal and equatorial tiers that normally contribute to the CNS but not to the retina are competent to form retina if they are moved to the dorsal-animal retinogenic region by experimental manipulations (Huang and Moody, 1993). Further, locally increasing or decreasing the activity of several signaling pathways demonstrated that the early restriction of retinogenic potential to the dorsal-animal quadrant depends upon the level of local BMP signaling experienced by a cell (Moore and Moody, 1999), consistent with the proposal that eye specific genes require a higher level of neural inductive signaling in order to form retinal tissue (Chow et al., 1999;Kenyon et al., 2001). However, there also is a vegetal repression of retinal fate in cleavage blastomeres (Huang and Moody, 1993;Moore and Moody, 1999); vegetal-tier blastomeres in the 32-cell embryo do not form retina when transplanted to the dorsal-animal retinogenic region, even when supplemented with neural, anteriorizing or dorsal mesoderm promoting signaling molecules (Fig. 1;Moore and Moody, 1999). It is particularly interesting that these manipulations do allow the vegetal-tier blastomeres to express neural fates, suggesting that acquiring a retinal fate requires additional information that is repressed in vegetal cells. In this study we set out to define which maternal molecules are responsible for this block of retinal competence.

In *Xenopus* several maternal molecules are enriched in the vegetal hemisphere and are involved in specifying the germ line, mesoderm and endoderm (reviewed in Sullivan et al., 1999). We investigated two of these (VegT, Vg1) because they promote mesodermal and endodermal fates, and thus might endogenously repress neural and retinal fates in vegetal cells. VegT is a T-box transcription factor that plays an essential role in the formation of mesoderm and endoderm (Zhang and King, 1996;Zhang et al. 1998;Clements et al., 1999;Kofron et al., 1999;Chang and Hemmati-Brivanlou, 2000;Xanthos et al. 2001). It appears to regulate endoderm formation by regulating members of Sox17, GATA, and Mix/Bix/Mixer families (Hudson et al., 1997;Henry and Melton, 1998;Tada et al., 1998;Casey et al., 1999;Clements et al., 1999;Weber et al., 2000;Xanthos et al., 2001) and to promote ventral mesoderm via Nodal signaling (Clements et al., 1999;White et al., 2002;Loose and Patient, 2004;Taverner et al., 2005). Vg1, a member of the TGF- β superfamily distinct from but closely related to the Nodal signaling proteins XNr1, XNr2, XNr4 and Derriere (Kimelman and Griffin, 2000;Xanthos et al., 2001;White et al., 2002), also appears necessary for dorsal mesoderm and endoderm formation (Kessler and Melton, 1995;Henry and Melton, 1998;Kessler, 1999). Thus, both molecules are in the right place at the right time and have an appropriate function to be candidates for the repression of retinal fates in vegetal blastomeres.

To examine whether VegT and/or Vg1 repress retinal fate three types of experiments were performed. First, the factors were over-expressed in dorsal-animal precursors of the CNS and retina to determine whether they repress these fates. Secondly, their endogenous levels were reduced in dorsal-animal blastomeres to determine whether neural and/or retinal fates were expanded. Third, their endogenous levels in ventral vegetal blastomeres were reduced to test whether this is sufficient to permit expression of neural and/or retinal fates at this ectopic position. We show that both VegT and Vg1 modulate the extent of neural/retinal fate in dorsal-animal lineages by converting cells from neural ectoderm to non-neural ectoderm, mesoderm

and endoderm fates. These effects appear to be mediated cell autonomously by *sox17a*, which converts the lineage to endoderm, and non-autonomously via *Derriere* signaling. The effects of both *VegT* and *Derriere*, but not *Vg1*, are reversed by co-expression of *notch1*, which is known to activate eye-specific genes (Kumar and Moses, 2001; Onuma et al., 2002), suggesting that those factors act in at least partially independent pathways. In contrast, reduction of *VegT* and/or *Vg1* in ventral vegetal blastomeres can allow these lineages to give rise to small numbers of neural progeny, but this is not sufficient to produce retinal tissue. Similarly, reduction of both BMP and Wnt signaling in the ventral vegetal lineages can give rise to neural but not retinal tissue (Moore and Moody, 1999). However, the combined repression of *VegT/Vg1* and BMP/Wnt in ventral vegetal cells leads to formation of retinal cells. Thus, in order for an animal lineage to become a precursor of the retina, it must not express endo-mesoderm promoting factors; for a vegetal lineage the correct, endogenous signaling environment must additionally be provided.

Materials and methods

Embryo collection and blastomere injection of mRNAs

Fertilized *Xenopus laevis* eggs were obtained by gonadotropin-induced natural matings of adult frogs and prepared for injection as described (Moody, 2000). mRNAs were synthesized in vitro and injected at the indicated concentrations: *tALK7* (2.0ng, Reissmann et al., 2001), *β-gal* (100pg), *tBR* (250pg, Graff et al., 1994), *cerberus-long* (*cer-L*; 200pg; Piccolo et al., 1999); *cerberus-short* (*cer-S*; 200pg; Piccolo et al., 1999), *derriere* (50pg; Sun et al., 1999), *cm-derriere* (500pg; Sun et al., 1999), *gfp* (200pg), an activated form of *notch1* (XNICD, 50-500pg, Coffman et al., 1993), *Xnr1* (25pg, Osada and Wright, 1999), *noggin* (50pg, Smith and Harland, 1992), *sox17a* (5-200 pg, Hudson et al., 1997), *VegT* (5-200pg, Zhang and King, 1996), an activated form of *Vg1* (*BVg1*; 5-50 pg, Joseph and Melton, 1998), *dnVg1* (100 pg, Joseph and Melton, 1998) and *dnWnt8* (500pg, Hoppler et al., 1996). Test mRNAs mixed with lineage tracer mRNA or lineage tracer mRNA alone were injected into one blastomere of the 16- or 32-cell embryo as described (Moody, 2000; Fig. 1).

Morpholino antisense oligonucleotides

An antisense *VegT* morpholino oligonucleotide (*VegTMO*) that is known to deplete maternal *VegT* (Zhang et al., 1998), the corresponding sense MO as a control (*cVegTMO*) and a standard control MO (*cMO*) were obtained commercially (Gene Tools). Blastomeres were injected with 16ng of MO mixed with lineage tracer (*β-gal* or *gfp*) mRNA.

Whole mount in situ hybridization

Embryos were fixed, stained for expression of the β -Gal lineage tracer and processed for whole mount in situ hybridization according to standard protocols (Sive et al., 2000) using Digoxigenin-labeled RNA probes for *foxD5* (Sullivan et al., 2001), *otx2* (Blitz and Cho, 1995), *sox3* (Penzel et al., 1997), *notch1* (Coffman et al., 1990), *rx1* (Mathers et al., 1997), *en2* (Hemmati-Brivanlou et al., 1991), *Krox20* (Bradley et al., 1993), *keratin* (Jonas et al., 1989), *Xbra* (Smith et al., 1991), *sox17a* (Hudson et al., 1997) and *endodermin* (*edd*) (Sasai et al., 1996). In some cases the widths of the expression domains of *sox3* on both the injected (β -Gal positive) and uninjected sides were measured with an eyepiece micrometer at 40X. The mean percent difference between sides was compared to similar measurements from lineage tracer injected, control embryos by the Student's t-test.

Cell fate analyses

Embryos were raised in Steinberg's solution, fixed in 4% paraformaldehyde, 3% sucrose in PBS at stage 37/38 (Nieuwkoop and Faber, 1994) and serially sectioned with a cryostat. Serial

sections were analyzed for the presence of GFP-labeled cells using epifluorescence microscopy. The distribution of GFP-positive progeny was compared to established fate maps (Moody, 1987a,b;Huang and Moody, 1993) and to control embryos injected with *gfp* mRNA. The frequencies that embryos contained labeled cells in different tissues in experimental and control groups were compared using the z-test of proportions as described (Moore and Moody, 1999). In some data sets, the size of the D1.1 contribution to the retina was estimated from the area of the retina containing GFP-labeled cells, as previously reported (Moore and Moody, 1999). To quantify effects on the size of the retina, its area was measured from serial tissue sections and volumes calculated as previously described (Moore and Moody, 1999). Measurements were compared between samples by the Student's t-test.

Blastomere transplantation

VegTMO (16ng) mixed with *gfp* mRNA (200pg) was injected bilaterally into the vegetal pole at the 2-cell stage. When embryos reached the 32- to 64-cell stage, one ventral vegetal blastomere (V2.1.1; Fig. 1) was transplanted to the position of the most retinogenic blastomere (* in Fig. 1) in an unlabeled host embryo as described (Huang and Moody, 1993;Moore and Moody, 1999;Moody, 1999). Embryos were cultured in Steinberg's solution plus gentamycin (1:1000) until stages 26-28 and fixed for cell fate analyses.

Results

Increased expression of either VegT or Vg1 in dorsal-animal blastomeres represses retina development

Previous studies demonstrated that ventral vegetal blastomeres are not competent to express a retinal fate, and predicted that this was due to the presence of an endogenous maternal factor (Huang and Moody, 1993;Moore and Moody, 1999). If endogenous VegT were involved, then increasing its expression in a major retina-producing blastomere should suppress retina formation. To test this, a series of doses of *VegT* mRNA was injected into one dorsal-animal blastomere (D1.1; Fig. 1) that normally produces about 60% of retinal cells (Huang and Moody, 1993). The eyes on the injected side of these embryos were smaller compared to those in *gfp* mRNA-injected control embryos (Fig. 2A, B), and the frequency of this eye defect positively correlated with the dose of injected *VegT* mRNA (51% at 5pg to 91% at 200pg). To verify that the retinal tissue was smaller the volumes of the retinas were measured; these were significantly reduced on the VegT-expressing side, whereas in GFP-expressing control embryos no significant difference was observed (Fig. 2B).

The reduced size of the retina could result from either death of the VegT-expressing cells or their conversion to a different fate. To distinguish between these possibilities, the GFP-labeled, VegT-expressing cells were fate mapped from serial tissue sections. First, no evidence of dying cells (as described in detail in Moody, 1999) was observed. Second, large numbers of differentiated GFP-labeled cells were found in the embryos, but their presence in the retina was dramatically reduced by VegT (Fig. 2C, E). Whereas 100% of GFP-injected control embryos contained abundant labeled D1.1 descendants in the retina, as expected from normal fate maps (Huang and Moody, 1993), VegT expression significantly reduced this frequency in a dose dependent manner (Fig. 2C). Additionally, the size of the D1.1 clone in the retina was reduced; for embryos injected with 5pg of *VegT* mRNA, the D1.1 retinal clones were about one-third the size of normal clones and for those injected with 100-200pg of *VegT* mRNA the D1.1 retinal clones consisted of less than 10 cells. Interestingly, effects on the D1.1 lineage were not confined to the retina. Fate mapping of D1.1 clones injected with 100pg *VegT* mRNA showed that this lineage, which normally contributes large numbers of cells to ectodermal derivatives and smaller number of cells to mesodermal or endodermal derivatives (Moody, 1987a), also contributed significantly less frequently to other neural structures (forebrain,

4.2%; midbrain, 8.3%; hindbrain and spinal cord, 37.5%; olfactory pit 0%; cranial ganglia, 8.3%) compared to controls (100% for all except cranial ganglia, 93.3%; $p < 0.05$). These clones contributed significantly more frequently to some mesodermal structures (e.g., pronephros, 41.7% vs. 6.2% in controls) ($p < 0.05$). The amount of endoderm populated by the VegT-expressing D1.1 clone appeared larger than in controls (Fig. 2F).

Expression of an activated form of Vg1 (*BVg1*, which contains a cleavage site necessary for protein activation; Joseph and Melton, 1998) in blastomere D1.1 also resulted in significantly smaller retinas (Fig. 2A, B); the effect often was bilateral as expected for a secreted protein. Vg1 reduced the frequency that the D1.1 lineage contributed to the retina in a dose-dependent manner (Fig. 2D, E). The size of the D1.1 clone in the retina also was reduced; for embryos injected with 12.5pg of *BVg1* mRNA, the D1.1 retinal clones were about one-quarter the size of normal clones and for those injected with 100-200pg of *BVg1* mRNA the D1.1 retinal clones contained less than 10 cells. Fate-mapping of Vg1-expressing D1.1 clones showed a significantly less frequent contribution to other neural structures (olfactory placode, 0%; forebrain, 10%; midbrain, 0%; hindbrain and spinal cord, 0%; cranial ganglia, 15%) compared to controls ($p < 0.05$), and the amount of endoderm populated by the Vg1-expressing D1.1 clone appeared larger than in controls (Fig. 2F). These results demonstrate that both VegT and Vg1 antagonize retinal fate, but these effects were not specific to the retina. Both factors also caused significantly smaller contributions to other neural tissues and enhanced contributions to the endoderm.

VegT and Vg1 both alter retinal fates by converting lineages to non-neural tissues

The fate mapping results indicate that VegT and Vg1 suppress neural/retinal fates and expand mesoderm and endoderm fates, consistent with several previous studies. To determine whether these effects are the result of alterations in germ layer induction, neural induction, retina-specific induction or neural plate patterning, the expression patterns of several genes during gastrulation and neural plate/neural fold stages were analyzed. VegT expression in the D1.1 lineage repressed two neural ectoderm genes that are expressed during gastrulation (*foxD5*, *otx2*), whereas genes expressed in non-neural ectoderm (*ker*), mesoderm (*Xbra*) and endoderm (*sox17a*, *edd*) were ectopically induced (Fig. 3A; Table 1). At later neural plate stages, the expression domains of two pan-neural plate genes (*sox3*, *notch1*) were smaller on the VegT-expressing side (Fig. 3B) in a significant number of embryos (Table 1); the mean width of the neural plate on the VegT-expressing side was reduced by 30% (*sox3*) and 28% (*notch1*) compared to β -Gal-expressing controls ($p < 0.001$). Region-specific gene expression patterns at neural plate/neural fold stages also were disrupted. Eye field (*rx1*), forebrain (*otx2*), midbrain (*en2*) and hindbrain (*Krox20*) genes were significantly repressed on the injected side (Fig. 3B; Table 1), suggesting that the effect is on early establishment of the neural ectoderm rather than regionalization of that tissue. Vg1 expression in the D1.1 lineage caused identical changes in all of the monitored genes except keratin; there was no evidence of ectopic or expanded non-neural ectoderm (Fig. 3A; Table 1). These results indicate that increased expression of either VegT or Vg1 in the major retina-producing lineage (D1.1) reduces the size of the neural ectoderm and promotes formation of the other germ layers during gastrulation; these early changes in fate subsequently affect the expression of later neural genes including one that is necessary for retina formation (*rx1*).

notch1 can reverse the repression of neural/retinal fate caused by VegT but not by Vg1

notch1 is expressed throughout the neural plate (Fig. 3B; Coffman et al., 1990), and there is evidence that it acts upstream of retinal-fate specifying genes (Kumar and Moses, 2001; Onuma et al., 2002). To verify that it has a neural/retinal fate promoting effect, we expressed an activated *notch1* construct (XNICD, truncated to include only the intracellular signaling domain; Coffman et al., 1993) in the D1.1 lineage. While it had no effect on the expression of

foxD5 or *en2*, it weakly induced other neural plate/neural fold markers and strongly induced *rx1* (Fig. 3; Table 1). Therefore, we tested whether the repression of neural and retinal fates in the D1.1 lineage by VegT or Vg1 could be reversed by co-expression of *notch1*. The frequency of the small eye phenotype caused by 100pg VegT mRNA was reduced by *notch1* mRNA in a dose-dependent manner (Fig. 4A), and the proportion of embryos in which VegT-expressing D1.1 progeny populated the retina was increased by co-expression of *notch1* in a dose-dependent manner (Fig. 4B). *notch1* reduced the frequency and extent of the VegT-induced repression of nearly all neural genes (*foxD5* and *Krox20* were not rescued), and reduced the ectopic induction of non-neural ectoderm (*ker*) (Fig. 3; Table 1). The mean width of the neural plate measured by *sox3* expression on the side injected with *VegT/notch1* mRNAs was only slightly reduced from controls (6.2%), and was significantly larger ($p<0.001$) than those injected with *VegT* mRNA alone. The *VegT/notch1* retina was significantly larger than the *VegT* alone retina, although it remained smaller than controls (Fig. 2B). Interestingly, *notch1* did not prevent the VegT-induced ectopic expression of *Xbra*, *sox17a* or *edd* (Fig. 3; Table 1), consistent with the observations that *notch1* alone did not affect the expression of non-neural ectoderm, mesoderm or endoderm markers (Table 1). These results indicate that most of the effects of VegT on both neural and non-neural ectoderm can be reversed through Notch1 signaling, whereas the VegT effects on mesoderm and endoderm are independent.

In contrast, the effects of Vg1 were not rescued by *notch1*. *Vg1/notch1*-expressing embryos still displayed the small eye phenotype at high frequency (Fig. 4C), their D1.1 progeny rarely populated the retina (Fig. 2D) and retinal volumes were not different from *Vg1*-injected embryos (Fig. 2B). Neither the repression of neural/retinal genes nor the ectopic expression of mesodermal and endodermal genes was changed compared to *Vg1*-injected embryos (Fig. 3; Table 1). These results indicate that the ability of Vg1 to repress neural/retinal fates is independent of Notch1 signaling, and therefore likely occurs via a pathway distinct from VegT.

Reduction of either VegT or Vg1 in dorsal-animal blastomeres expands the neural/retinal field

Although VegT mRNA is enriched in the vegetal pole (Lustig et al. 1996; Stennard et al., 1996; Zhang and King, 1996; Horb and Thomsen, 1997), its protein product is proposed to form a concentration gradient from vegetal to animal poles (Kavka and Green, 1997; Green, 2002) and low levels of maternal RNA are reported in animal caps (Stennard et al., 1996). To test whether VegT endogenous to animal blastomeres limits neural/retinal fate, VegT protein was locally reduced by injecting a VegT antisense morpholino (VegTMO) unilaterally into the dorsal-animal region at the 2-cell stage. The retinas on the VegT-depleted side were significantly larger than those on the uninjected side, whereas no differences were observed in control embryos injected with either control MO (cVegTMO, cMO; Fig. 5A, B). In all embryos, large numbers of VegTMO-injected cells contributed to the retina (Fig. 5A). Interestingly, the retinal volume on the uninjected side of VegT-depleted embryos was significantly larger than those in control embryos (Fig. 5B), likely due to the fact that the retina derives cells from both sides of the embryo (Jacobson and Hirose, 1978; Huang and Moody, 1993; see green cells on uninjected side of Fig. 5A). Other regions of the CNS also appeared enlarged in dorsal-animal VegT-depleted embryos (note thicker forebrain in Fig. 5A). This was confirmed by analyzing the expression domains of both pan-neural plate and retinal genes. The domains of *sox3*, *notch1* and *rx1* were larger on the VegT-depleted side of embryos (Fig. 5C; Table 1); the mean width of the *sox3* (30%) and *notch1* (16%) domains were significantly expanded ($p<0.05$), whereas β -gal mRNA, cMO or cVegTMO did not alter these domains. Because dorsal-animal depletion of VegT did not alter the endogenous domains of mesodermal and endodermal genes (Fig. 5D; Table 1) that could alter the extent of neural induction, the effects on neural/retinal markers appear to be autonomous to the ectoderm.

To reduce endogenous dorsal-animal Vg1 activity, dnVg1, which reduces both dorsal mesoderm and endoderm development (Joseph and Melton, 1998), was expressed in the D1.1 lineage on one side of the embryo. It caused enlargement of the retina on the injected side (Fig. 5A, B) and in all embryos large numbers of *dnVg1*-expressing cells populated the retina on the injected side (Fig. 5A). Both pan-neural plate and retinal markers were expanded, whereas in no embryo was ectopic *sox17a* or *edd* induced (Fig. 5C, D; Table 1). However, *Xbra* expression was reduced in a significant number of embryos.

These results indicate that both endogenous VegT and endogenous Vg1 in the dorsal-animal lineage restrict the extent of both neural and retinal progeny. The VegT and Vg1 gain-of-function (GOF) and loss-of-function (LOF) phenotypes described above are very similar, suggesting that they may act through the same pathway. Although there is no evidence to date that VegT acts through Vg1 signaling, it is known that they both can affect the expression of several similar downstream targets (see next section). Several experiments were performed to determine if these two factors work in concert to affect retinal fate. First, *VegT* and *BVg1* mRNAs were co-expressed in the D1.1 lineage. For nearly every marker gene analyzed the frequency of the effect was increased over that observed after expression of only one of the mRNAs (Table 1). The exceptions were: 1) for two markers (*foxD5*, *Xbra*) the effects were already near to 100% for the single mRNA injections; and 2) for *keratin* the phenotype followed the Vg1 effect (no change) rather than the VegT effect (expansion). Co-expression of VegT and Vg1 also significantly reduced the size of both retinas compared to each factor alone (Fig. 2B). Second, both VegT and Vg1 were depleted in the D1.1 lineage (VegTMO/dnVg1); *sox3*, *notch1* and *rx1* expression domains were expanded at a significantly higher frequency compared to depletion of only one factor (Fig. 5C; Table 1). However, the size of the retina did not increase compared to VegTMO alone (Fig. 5A, B; $p > 0.05$), perhaps because single depletion already resulted in maximum tissue expansion. Third, we examined whether each construct could rescue the retinal phenotype of the other. VegT LOF in the D1.1 lineage via injection of VegTMO expanded *rx1* expression (Fig. 5C; Table 1); this phenotype was not altered by co-expression of *Vg1* (Fig. 5E; 43.8%, $n=48$). Vg1 LOF in the D1.1 lineage via expression of *dnVg1* mRNA also expands *rx1* expression (Fig. 5C; Table 1); this phenotype was reversed by co-injection of *VegT* mRNA (Fig. 5E; 71.7%, $n=53$). Conversely, VegTMO rescued the Vg1 small eye phenotype but dnVg1 did not rescue the VegT small eye phenotype (Fig. 5F). These results indicate that VegT can compensate for loss of Vg1, but Vg1 does not restore VegT LOF. These observations further support the idea generated from the *notch1* rescue experiments that VegT and Vg1 likely regulate early neural/retinal fates in dorsal-animal lineages via partially over-lapping but not identical molecular pathways.

Repression of neural/retinal fate results from a combination of Sox17 α and Derriere expression

VegT is known to act through two downstream pathways: 1) endoderm formation is promoted via regulation of *sox17 α* (Hudson et al., 1997; Clements et al., 1999; Yasuo and Lemaire, 1999; Clements and Woodland, 2003); and 2) mesoderm formation is promoted via activation of TGF β signaling involving in particular XNr1-2, XNr4-6 and Derriere (Clements et al., 1999; Sun et al., 1999; Yasuo and Lemaire, 1999; Kimelman and Griffin, 2000; Takahashi et al., 2000; Xanthos et al., 2001; Rex et al., 2002; White et al., 2002; Sinner et al., 2004). There also is evidence that Vg1 can induce XNr1-2, XNr4 and Derriere (Sun et al., 1999; Agius et al., 2000; Hanafusa et al., 2000). To discern which of the common potential downstream genes might mediate the VegT/Vg1 repression of neural/retinal fate some of the individual components were expressed in the D1.1 lineage.

In *sox17a* -injected embryos, retinas developed in all cases, although often they developed deep within the embryo rather than at the surface (Fig. 6A). The volumes of these retinas were

not different from controls (Fig. 6B; $p > 0.05$), but they rarely contained *sox17a*-expressing cells (Fig. 6A, C). The sizes of the expression domains of *sox3*, *notch1* and *rx1* were not altered ($p > 0.05$, Fig. 6D; Table 1). However, lineage-labeling demonstrated that these tissues were derived from the adjacent, uninjected blastomeres (i.e., not β -Gal-positive). Thus, although *sox17a* expression in the D1.1 lineage prevents descendants from populating the retina (Fig. 6C), the remaining lineages are able to compensate to produce normal-sized neural plates and retinas. Consistent with previous studies (Hudson et al., 1997), *sox17a* reduced the expression domain of *Xbra* and expanded the expression domain of *edd* at the sites of the injected clones (Fig. 6D; Table 1). Thus, increased expression of *sox17a* converts the D1.1 lineage from a predominantly neural/retinal fate to a predominantly endodermal fate. This reproduces the expansion of endoderm by VegT and Vg1 but does not account for their alterations of neural and retinal fates. Therefore we investigated the effects of some of the Nodal-related molecules.

In *derriere*-injected embryos, the volumes of the retinas on both sides of the embryo were dramatically smaller than controls (Fig. 6A, B); these bilateral effects are expected of a secreted protein. Like VegT and Vg1 embryos, the retinas only rarely contained *derriere*-expressing D1.1 progeny (Fig. 6A, C). *derriere* also caused a significant reduction in the size of the expression domains for early neural, pan-neural plate ($p < 0.05$ for *sox3*, *notch1*) and region-specific neural genes (Fig. 6D; Table 1). Like VegT- but not like Vg1-injected embryos, *derriere* caused ectopic *keratin* expression. Like both VegT and Vg1 embryos, *derriere* caused ectopic or expanded domains of mesodermal and endodermal genes (Fig. 6D; Table 1). Thus, *derriere* GOF in the D1.1 lineage phenocopies the VegT effect and mostly reproduces the Vg1 effect. In *Xnr1*-injected embryos, the mean size of the retina was indistinguishable from controls (Fig. 6A, B), even though the D1.1 progeny expressing *Xnr1* rarely populated the retina (Fig. 6C). Like VegT and Vg1 embryos, *Xnr1* caused ectopic or expanded expression of mesodermal and endodermal genes (Fig. 6D; Table 1), but like Vg1, it had no effect on *keratin* expression (Table 1). *Xnr1* effects on the neural/retinal genes were pleiotropic; domains could either reduced or expanded (Fig. 6D; Table 1). This was never observed with VegT or Vg1, suggesting it resulted from the known differential effects of *Xnr1* on the mesoderm (Clements et al., 1999; Kofron et al., 1999; Agius et al., 2000). Together these results indicate that *derriere* is sufficient to mediate the VegT and possibly Vg1 effects on dorsal-animal lineages.

We further tested whether the *derriere* effects could be attributable to VegT versus Vg1 by determining whether they could be rescued by co-expression of *notch1*. Both the retinal volume and the proportion of embryos in which *derriere*-expressing D1.1 progeny populated the retina were significantly increased after *notch1* co-injection (Fig. 6B, C; cf. *derriere* alone). Interestingly, *notch1* rescued the retinal phenotypes on both sides of the embryo even though it is not secreted (Fig. 6A, B), suggesting that *notch1* may either promote the expression of a secreted factor that antagonizes *Derriere* or interfere with *Derriere* upstream of its secretion. These points will require further investigation. Co-expression of *notch1* also significantly reduced the repressive effect of *derriere* on most neural and retinal expression domains (Table 1); like *VegT/notch1*-injected embryos, there was no rescue of the *foxD5* repression. Like *VegT/notch1*-injected embryos, the ectopic induction of *keratin* was reversed whereas there was no effect on the *derriere*-mediated expansion of mesodermal and endodermal genes (Fig. 6D; Table 1). These data indicate that the *derriere* effects are likely part of the VegT pathway independent of Vg1. In contrast, the effects of *Xnr1* on the D1.1 lineage are not rescued by *notch1*. Retinal volume was unchanged and *Xnr1/notch1*-expressing cells rarely populated the retina (Fig. 6B, C). *notch1* did not alter the pleiotropic effects on the *sox3* and *rx1* expression domains nor prevent the *Xnr1*-induced expansion of mesodermal and endodermal genes (Fig. 6D; Table 1).

These results were confirmed by LOF experiments. Expression of a dominant-negative Derriere construct (*cm-derriere*), which has minimal effects on XNr1, XNr2, XNr4 or Vg1 signaling (Sun et al., 1999), in the D1.1 lineage caused enlargement of the retina on both injected and uninjected sides (Fig. 6A, B). In all embryos *cm-derriere*-expressing D1.1 progeny densely populated the retina (Fig. 6A, C), and pan-neural plate and retinal markers were expanded (Fig. 6D; Table 1). *Xbra* expression was reduced but endoderm markers were not altered. The neural/retinal results phenocopy both *VegT* and *Vg1* LOF, whereas the effects on *Xbra* are most similar to those of *dnVg1* (Fig. 5D; Table 1). Expression of a dominant-negative XNr1 receptor (tALK7) in the D1.1 lineage did not phenocopy either *VegT* or *Vg1* LOF. As with *Xnr1* GOF, the effects on pan-neural plate and retinal markers were pleotropic (Table 1). *Xbra* expression was significantly repressed, consistent with the proposed function of XNr1 in mesoderm formation (Osada and Wright, 1999; Agius et al., 2000).

Together the GOF, Notch1 rescue and LOF experiments indicate that Derriere is the most likely Nodal-related mediator of either *VegT* or *Vg1* effects on the dorsal-animal lineages. However, reversal of its effects by Notch1 suggests that in this system it likely is predominantly activated by *VegT*. Our data do not support a strong role for XNr1 in mediating the effects of *VegT* or *Vg1* on D1.1 neural/retinal fates because it did not closely phenotype either gene. However, both *VegT* and *Vg1* can up-regulate the expression of several other Nodal-related genes (Agius et al., 2000; Takahashi et al., 2000; Rex et al., 2002), which could play a role in the phenotypes that our experiments have described. Nonetheless, the above data show that Derriere is sufficient to cause all of the described effects. In addition, *sox17a* likely contributes to the repression of neural/retinal fates in both *VegT* and *Vg1* pathways by converting cells to endoderm in a cell-autonomous manner. But, *sox17a* expression alone does not account for the non-autonomous effects on neural/retinal fates; a secreted factor such as Derriere is implicated.

To confirm a role for Derriere as a downstream mediator of the *VegT* effect on neural/retinal fates, we performed two experiments. Co-injection of *VegTMO* and *derriere* mRNA in the D1.1 lineage reversed the *VegTMO*-induced expansion of *sox3*, *notch1* and *rx1* expression domains to significant repression (Fig. 6E; Table 1). Likewise, co-injection of *cm-derriere* mRNA with *VegT* mRNA reversed the *VegT*-induced reduction of these three neural/retinal genes (Fig. 6E; Table 1). These results indicate that Derriere acts downstream of *VegT* to mediate its effects on neural/retinal fates.

Depletion of endo-mesodermal factors alone in ventral-vegetal blastomeres is not sufficient to induce retinal fates

Previous studies indicated that vegetal blastomeres are not competent to form retina even when transplanted to a retinogenic region of the embryo or when supplemented with neuralizing, anteriorizing or dorsal mesoderm-promoting factors that allow the lineage to express a neural fate (Huang and Moody, 1993; Moore and Moody, 1999). These studies predicted that a vegetal-localized maternal factor is responsible for this inhibition of retinal fate. *VegT* and *Vg1* are both expressed at high levels in the vegetal lineages, and the experiments described above demonstrate that each promote mesodermal and endodermal fates at the expense of neural/retinal fates in dorsal-animal lineages. If these factors were singularly responsible for blocking retinogenic competence of vegetal blastomeres in which their endogenous levels are relatively high, then reducing their activity in the vegetal hemisphere should be sufficient for vegetal lineages to produce retinal cells.

This was tested by reducing *VegT* and/or *Vg1* levels in ventral vegetal blastomeres and assaying for the presence of both neural and retinal descendants in the affected lineages. Confirming previous studies (Xanthos et al., 2001), gut development was dramatically reduced in embryos in which *VegT* was depleted throughout the vegetal hemisphere by *VegTMO*

injection at the 2-cell stage (not shown). Confining VegT depletion to a single ventral vegetal blastomere (V2.1.1; Fig. 1) likewise resulted in reduced expression domains of *Xbra* (87%, n=31), *sox17a* (84%, n=31) and *edd* (92%, n=36) (Fig. 7A), and the extent of GFP-labeled V2.1.1 progeny in the endoderm appeared reduced in tail bud embryos (Fig. 7B). To test whether the VegTMO-injected V2.1.1 lineage was converted to neural or retinal fates, embryos were assayed for ectopic *sox3* and *rx1* expression at neural plate stages; no cases of ectopic domains were observed (Fig. 7C; Table 2). Similar data were obtained when V2.1.1 was injected with either *dnVg1* mRNA or a combination of VegTMO/*dnVg1* mRNA (Fig 7C; Table 2). To determine whether small numbers of V2.1.1 progeny were converted to neural/retinal fates but incorporated into the endogenous tissues (i.e., not ectopically induced), a fate map of GFP-labeled cells in tissue sections from stage 37/38 embryos was constructed. Small numbers of GFP-labeled cells (~10-20/embryo) were observed infrequently in the caudal spinal cords of VegTMO-, *dnVg1*- and VegTMO/*dnVg1*-injected embryos (Table 2). However, none of these manipulations caused the V2.1.1 progeny to contribute to the retina (Table 2). Therefore, while depletion of these two factors can result in small numbers of cells becoming neural, it is not sufficient to promote a retinal fate in the V2.1.1 lineage. To test whether an external neurogenic environment would promote a retinal fate in these cells, V2.1.1 blastomeres that had been injected with VegTMO were transplanted to the most neurogenic/retinogenic region of the 32-cell embryo (* in Fig.1). In no case did the V2.1.1 cells populate the retina (n=14), indicating that the signaling environment within the V2.1.1 lineage needs to be altered cell-autonomously.

Previous work demonstrated that in order for the V2.1.1 lineage to express a retinal fate, three signaling pathways (Nodal, BMP and Wnt) need to be repressed (Moore and Moody, 1999). Although vegetal blockade of both Wnt and BMP signaling can cause a secondary head containing eyes to form (Glinka et al., 1997; Piccolo et al., 1999), lineage analysis demonstrated that the vegetal clone contributed sparsely to neural but not at all to retinal tissue (Moore and Moody, 1999). Only the combined repression of Wnt, BMP and Nodal signaling via expression of the long form of Cerberus (Cer-L) caused the injected V2.1.1 lineage to produce retinal progeny (Moore and Moody, 1999). We confirmed these results by showing that Cer-L induced ectopic *sox3* and *rx1* expression, and that the labeled V2.1.1 clone was part of the ectopic *rx1* domain (Fig. 7C; Table 2). In contrast, repression of both BMP and Wnt signaling (via co-expression of *nog/tBR/dnWnt8* mRNAs) also induced ectopic *sox3* and *rx1* expression (Table 2), but the V2.1.1 lineage did not contribute to the ectopic *rx1* domain (Fig. 7C). Fate mapping at later stages revealed that GFP-labeled V2.1.1 progeny contributed to the spinal cord in both *Cer-L*- and *nog/tBR/dnWnt8*-injected embryos, but they contributed to the retina only in *Cer-L* embryos (Table 2). Presumably the difference in effects between *Cer-L* and *nog/tBR/dnWnt8* mRNA injections is the additional ability of Cer-L to inhibit Nodals (Glinka et al., 1997; Piccolo et al., 1999). Therefore we tested whether blocking Nodal signaling either alone (using the short form of Cerberus [Cer-S] or various dominant-negative constructs [cm-Xnr2, cm-derriere]) or in various combinations with VegTMO induced a retinal fate. None of the combinations was sufficient to induce ectopic *rx1* expression or cause the V2.1.1 lineage to contribute to the retina (Fig. 7C; Table 2).

In contrast, inhibition of VegT in combination with blocking both BMP and Wnt signaling (via injection of VegTMO plus *nog/tBR/dnWnt8* mRNAs) induced a retinal fate as frequently as did Cer-L; ectopic domains of *rx1* expression were induced within the β gal-expressing V2.1.1 clone and V2.1.1 descendants populated the differentiating retina (Fig. 7C; Table 2). Combining VegTMO with blockade of only one signaling pathway did not cause this effect (Table 2). Interestingly, additional inhibition of Vg1 in this combination significantly increased the frequency of both ectopic *sox3* and *rx1* expression and contribution to both the spinal cord and the retina by V2.1.1 descendants (Fig. 7C; Table 2). These experiments confirm that while blocking both BMP and Wnt pathways in vegetal cells can transfect the descendants of these

cells to produce neural cells, it is not sufficient to transfect them to retina. Likewise, inhibiting endogenous endo-mesodermal factors (VegT/Vg1) can transfect the vegetal lineage to neural but not retinal. A retinal fate requires both sets of factors to be concomitantly reduced.

Discussion

An important question in development is how and when do pluripotent embryonic cells acquire the differential competence to contribute to specific tissues. Is this a stochastic process, or do early factors bias or restrict the selection of embryonic precursors? This question is readily approachable for retinal fate in *Xenopus* because each retina descends from a subset of nine animal blastomeres at the 32-cell stage, and each of these blastomeres produces characteristic proportions of the retina (Huang and Moody, 1993). Previous work demonstrates that the earliest steps in retina fate specification occur well before the overt morphogenesis of the eye cup (reviewed in Williams and Moody, 2004; Zaghoul et al., 2005). A first step selects a subset of cells to be competent to contribute to the retina (Fig. 1). Transplantation experiments showed that many blastomeres, not just the nine that normally produce the retina, are competent if moved to the appropriate retinogenic environment, i.e., the dorsal-animal quadrant. Thus, the potential to express a retinal fate may simply require the appropriate signaling environment for anterior neural tissues. However, vegetal blastomeres transplanted to this position never contribute progeny to retina (Huang and Moody, 1993). This developmental restriction is not overcome by providing components of signaling pathways involved in neural and anterior fate specification, even under conditions in which ectopic neural and retinal tissues are induced (Moore and Moody, 1999). These results predict that vegetal blastomeres contain one or more maternal molecules that specifically repress the competence of a cell to form the retina. Two maternal factors, VegT and Vg1, are candidates for the repression of retinal competence because both are vegetally-enriched, and both play essential roles in the formation of mesoderm and endoderm (Fig. 8). In this study we demonstrate that both factors negatively regulate the competence of cleavage blastomeres to produce neural and retinal lineages.

In dorsal-animal lineages, VegT and Vg1 affect the allocation of cells into the neural ectoderm

Several reports concur that VegT induces: 1) endoderm in a cell autonomous manner via Bix/Mix/Sox17 transcription factors; and 2) mesoderm via the activation of TGF β signals (Tada et al. 1998; Casey et al., 1999; Clements et al., 1999; Yasuo and Lemaire, 1999; Sun et al., 1999; Agius et al., 2000; White et al., 2002; Clements and Woodland, 2003; Loose and Patient, 2004; Taverner et al., 2005). Vg1 is thought to induce endoderm via induction of *sox17a*, *mix1* and *eomes* (Clements et al., 1999) and mesoderm via *mix* and *Xnrs* (Mead et al., 1998; Loose and Patient, 2004). We demonstrate that increased expression of either VegT or Vg1 in a retinogenic lineage represses the formation of the retina, an easily identifiable part of the anterior neural plate whose precursor blastomeres have been quantitatively mapped in the cleavage embryo (Fig. 1). However, by analyzing a large number of marker genes expressed at different times in early neural development, as well as fate maps of embryos containing differentiated tissues, we conclude that this repression is not specific to the retina, but affects the allocation of cells during gastrulation to the various germ layer domains. Markers of the earliest induced neural ectoderm are repressed and for VegT, but not Vg1, non-neural ectoderm is expanded. In addition, markers of early mesoderm and endoderm are expanded or ectopically expressed, consistent with previous reports (Kessler and Melton, 1995; Zhang and King, 1996; Clements et al., 1999; Kessler, 1999). Thus, these two factors both appear to affect retinal fate indirectly by repressing specification of cells to a neural ectodermal fate. Although this might be accomplished by conversion of cells directly to endo-mesodermal fates via expression of the known downstream target genes, there also may be involvement of two maternal *sox* genes (*sox3* in the animal hemisphere and *sox7* in the vegetal hemisphere) that are proposed to competitively regulate the *Xnr* genes to define the germ layer domains (Zhang et al., 2004;

2005). Since VegT and Vg1 also regulate *Xnr* gene expression, it will be important to determine if their downstream effects intersect with those of the maternal *sox* genes to allocate the extent of the neural ectodermal domain.

The VegT and Vg1 effects are likely mediated primarily by Sox17 α and Derriere

As noted above, both VegT and Vg1 can affect cell fate decisions by promoting mesoderm, via Nodal-related factors including XNr1-2, XNr4-6 and Derriere, and endoderm, via factors including Sox17 α . We tested some of these factors for potential involvement in the VegT/Vg1 effects on the dorsal-animal lineages. Part of the VegT/Vg1 GOF phenotypes can be attributed to their ability to induce *sox17 α* , and *edd* is a direct target of both Sox17 α and VegT (Engleka et al., 20001; Clements et al., 2003; Ahmed et al., 2004; Taverner et al., 2005). We demonstrate that exogenous expression of *sox17 α* in the dorsal-animal lineage mimics part of the VegT/Vg1 phenotype in a cell-autonomous manner; it converts the lineage from neural/retinal to endodermal, consistent with its initial description (Hudson et al., 1997). This aspect of the VegT/Vg1 phenotype also may involve other endodermal target genes; for example, expression of *bix1* in dorsal-animal cells represses head development and diverts cells from the notochord (Tada et al., 1998). However, similar analyses are not yet available for *bix4*, *edd*, *mix1*, *omes* or *sox7*, the latter of which also regulates *edd* (Zhang et al., 2005).

Although Sox17 α converted the dorsal-animal lineage from neural/retinal to endodermal, these embryos contained normal sized retinas and normal expression domains of neural and retinal genes. These results indicate that signaling factors that affect cells derived from uninjected neighboring blastomeres must be involved in the VegT/Vg1 phenotypes. This interpretation is consistent with the observation that neighboring blastomeres signal each other to reconstitute a normal sized retina after the major retinogenic blastomere is ablated (Huang and Moody, 1993), and with the fact that VegT and Vg1 induce several TGF β /Nodal signaling proteins (Clements et al., 1999; Kofron et al., 1999; Sun et al., 1999; Yasua and Lemaire, 1999; Agius, 2000; Xanthos et al., 2001; Eimon and Harland, 2001; Onuma et al., 2002; White et al., 2002; Loose and Patient, 2004). We tested two of these, Derriere and XNr1, for their ability to phenocopy the VegT/Vg1 phenotypes. The non-autonomous effects on the dorsal-animal lineage appear to be mediated primarily by Derriere because both its ectopic expression and inhibition mimicked the neural/retinal phenotypes of both VegT and Vg1. In contrast, results from similar experiments with XNr1 were very pleiotropic, indicating that this Nodal-related factor is not significantly involved. Further, Derriere GOF and LOF constructs reverse the effects of VegT LOF and GOF constructs, respectively, indicating it acts downstream of VegT. Interestingly, *derriere* has been shown to be a direct target of VegT (White et al., 2002; Taverner et al. 2005), although its transcription is also regulated by other Tbox proteins and other TGF β signaling factors (White et al., 2002).

The above description implies that manipulations of Derriere levels alone account for the VegT/Vg1 effects on dorsal-animal blastomere fate, but several caveats should be noted. First, *derriere* caused a greater repression of *sox3* and *rx1* than either VegT or Vg1, and a greater expansion of *sox17 α* . Second, Vg1, VegT and another maternal transcript factor whose function it regulates, Sox7, each induce several XNr factors (Clements et al., 1999; Kofron et al., 1999; Yasuo and Lemaire, 1999; Takahashi et al., 2000; Xanthos et al., 2001; Rex et al., 2002; Clements and Woodland, 2003; Zhang et al., 2005), and the other XNrs and Derriere cooperatively regulate each other (Eimon and Harland, 2002). There also is evidence that Derriere can dimerize with other XNr proteins, so that its effects may depend on the availability of these other related factors (Agius et al., 2000; Eimon and Harland, 2002). Thus, although our experiments support the notion that Derriere, and not XNr1, is sufficient to account for the VegT/Vg1 phenotypes in the dorsal-animal lineage, the effects downstream of Derriere and its interactions with the other XNr family members need to be further investigated.

The VegT and Vg1 pathways affecting neural/retinal fate are not identical

Although there are several similarities between the VegT and Vg1 phenotypes, as expected from the many common downstream factors that they regulate, there are some distinct differences. First, the effects of both VegT and Derriere can be reversed by Notch1, whereas neither Vg1 nor XNr1 effects are altered. There are several potential mechanisms by which Notch1 may reverse VegT/Derriere effects. First, Notch1 may specifically promote retinal fate (Fig. 8); in fly and *Xenopus*, Notch1 signaling promotes eye gene expression (Kumar and Moses, 2001; Onuma et al., 2002). Second, Notch1 may interfere with the induction of endodermal genes and thus be permissive to alternate fates; in zebrafish, Notch1 activation down-regulates endodermal genes in the marginal zone (Kikuchi et al., 2004). Third, Notch1 is well known in numerous systems to prevent differentiation and hold cells in an immature state (Chiba, 2006); it may delay mesodermal induction and thus allow cells to remain competent to respond to later expressed neural inducers. Interestingly, a recent study identified three Notch1 regulated genes as direct targets of VegT but did not identify Notch1 as a down-regulated gene (Taverner et al., 2005), suggesting that the potential interaction between the VegT and Notch1 pathways is indirect, perhaps via Derriere (Fig. 8). Other differences between VegT and Vg1 are that their increased expression has different effects on *keratin*, and their decreased expression has different effects on *Xbra*. Finally, Vg1 can not rescue the VegT LOF retinal phenotype, whereas VegT reverses the Vg1 LOF retinal phenotype. These data support a model of two endo-mesodermal pathways that regulate blastomere competence to produce retina that likely interact and are partially redundant (Fig. 8). Redundancy has also been noted in several studies reporting that VegT and Vg1 synergize to regulate endo-mesodermal fates (Clements et al., 1999; Yasuo and Lemaire, 1999; Agius et al., 2000).

For vegetal lineages to produce retinal progeny multiple endogenous molecules must be inhibited

In their simplest interpretation, the results presented herein demonstrate that if cleavage blastomeres express high levels of endo-mesodermal factors, then they express these tissues dominantly, which by default prevents the expression of neural/retinal fates. If this is the extent of the regulation of neural/retinal fate competence, then to produce retinal precursors from pluripotent embryonic cells, one need only deplete the cells of these factors. This scenario appears to hold for dorsal-animal blastomeres. However, experiments in vegetal cells show that the requirements for inducing neural and retinal fates are not equivalent and that simple depletion of endo-mesodermal factors is not sufficient to impose a retinal fate. Although VegT/Vg1 reduction allowed neural cells to develop within the vegetal lineage, in order for retinal cells to develop both BMP and Wnt pathways also needed to be reduced. Although this might be explained by the fact that anterior neural structures require prolonged BMP blockade (Hartley et al., 2001), transplantation experiments demonstrate that it is not sufficient to move a VegT depleted cell to the native neurogenic/retinogenic region of the embryo. In our hands the BMP and Wnt pathways needed to be inhibited within the vegetal lineage itself, suggesting that some component of the BMP/Wnt signaling that represses retinal fate is autonomous to the vegetal cell. Thus, the requirements for expressing a retinal fate are differentially allocated: dorsal-animal blastomeres can be converted to neural and retinal fates simply by reducing endomesodermal factors, whereas reducing these factors in vegetal blastomeres only minimally converts cells to a neural fate and does not produce a retinal fate unless combined with endogenous repression of both BMP and Wnt signaling.

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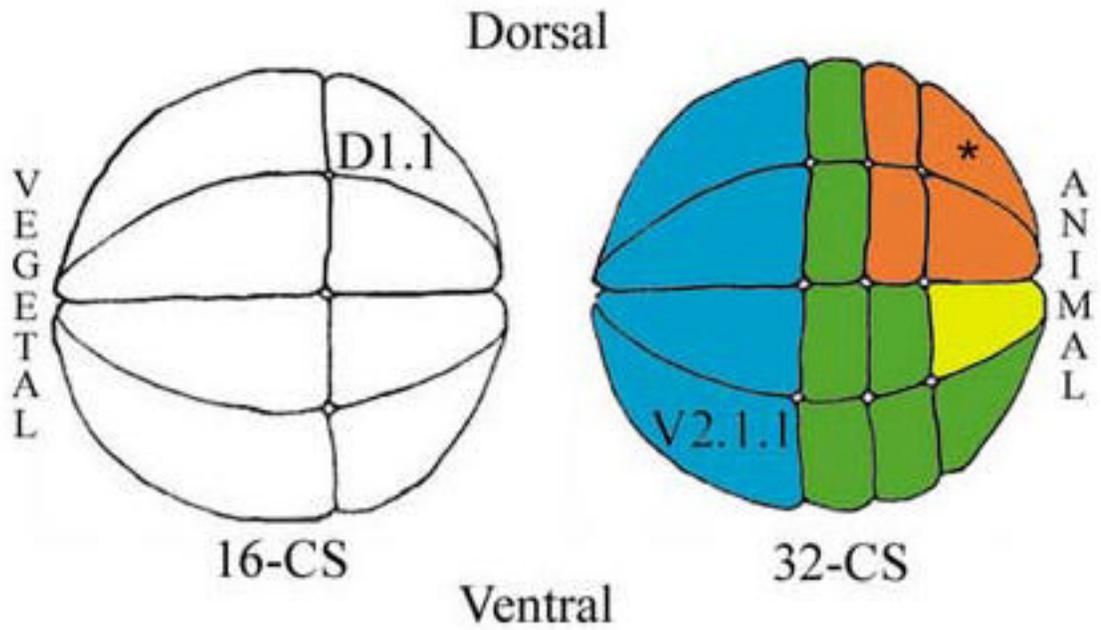
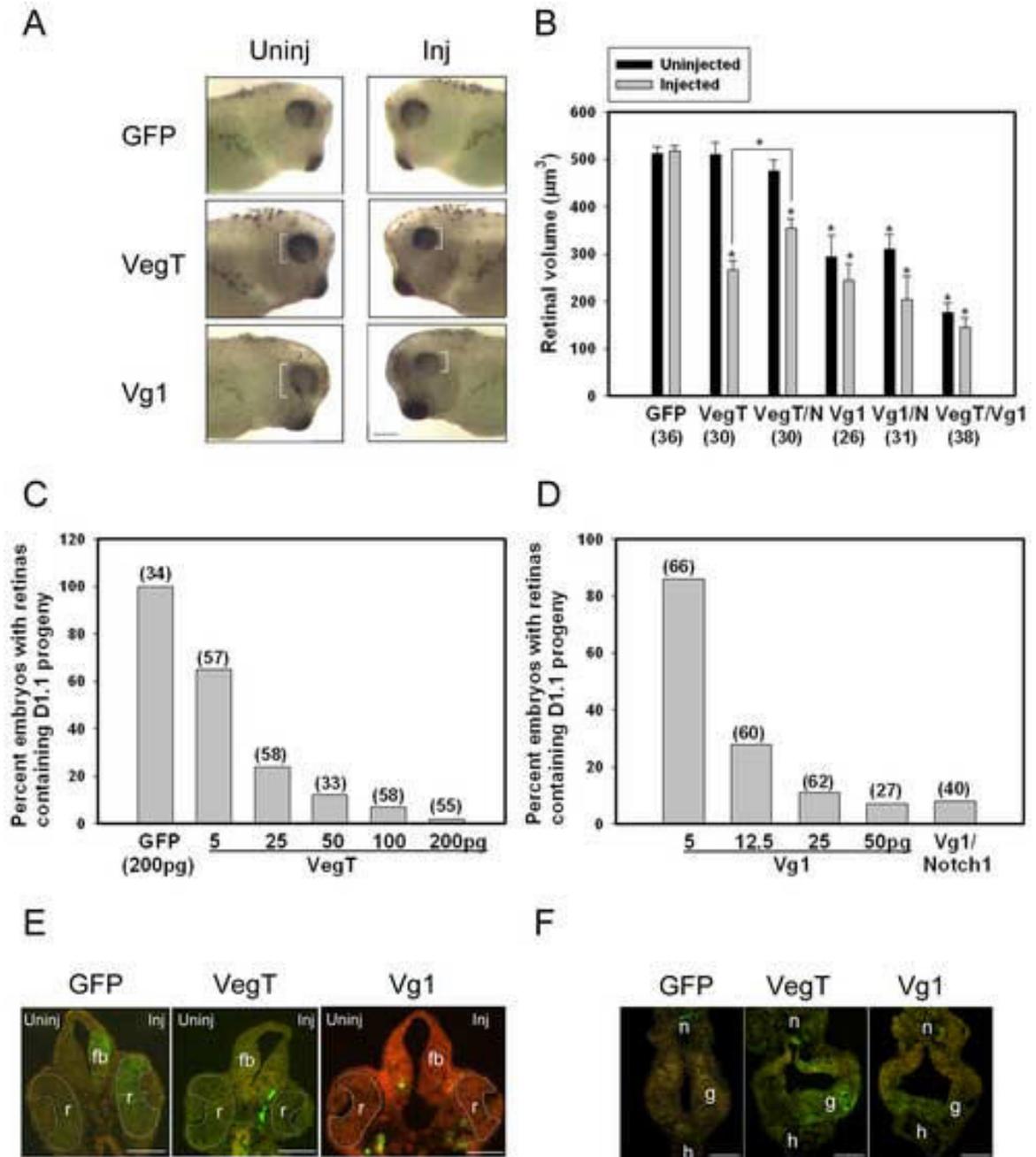


Figure 1.

Side views of 16- and 32-cell stage (CS) *Xenopus* embryos noting the positions of the two blastomeres manipulated in the described experiments (D1.1, V2.1.1). The 32-cell embryo shows four of the eight blastomeres that are highly retinogenic (orange), and the one that is slightly retinogenic (yellow); asterisk indicates the most retinogenic cell. Several other blastomeres are competent to form retina but normally do not (green) and vegetal blastomeres are not competent to make retina (blue).

**Figure 2.**

VegT and Vg1 inhibit the retinal fate of the dorsal-animal (D1.1) blastomere.

(A) Expression of *VegT* or *Vg1* in the D1.1 lineage results in a smaller eye (white brackets). Uninj, uninjected side; Inj, injected side. Scale bar = 400µm.

(B) The mean volume of retinas in embryos in which the indicated mRNAs (GFP, *VegT*, *Vg1*, notch1 [N]) were expressed. Numbers in parentheses indicate size of sample. * indicates $p < 0.01$ compared to GFP controls. *VegT* and *Vg1* both significantly reduced the size of the retina on the injected side; *Vg1* additionally reduced the retina on the uninjected side. Co-injection of *notch1* significantly increased the retinal volume of *VegT*-injected embryos, compared to *VegT* alone (bracket, $p < 0.01$), although they remained smaller than the GFP controls. Co-

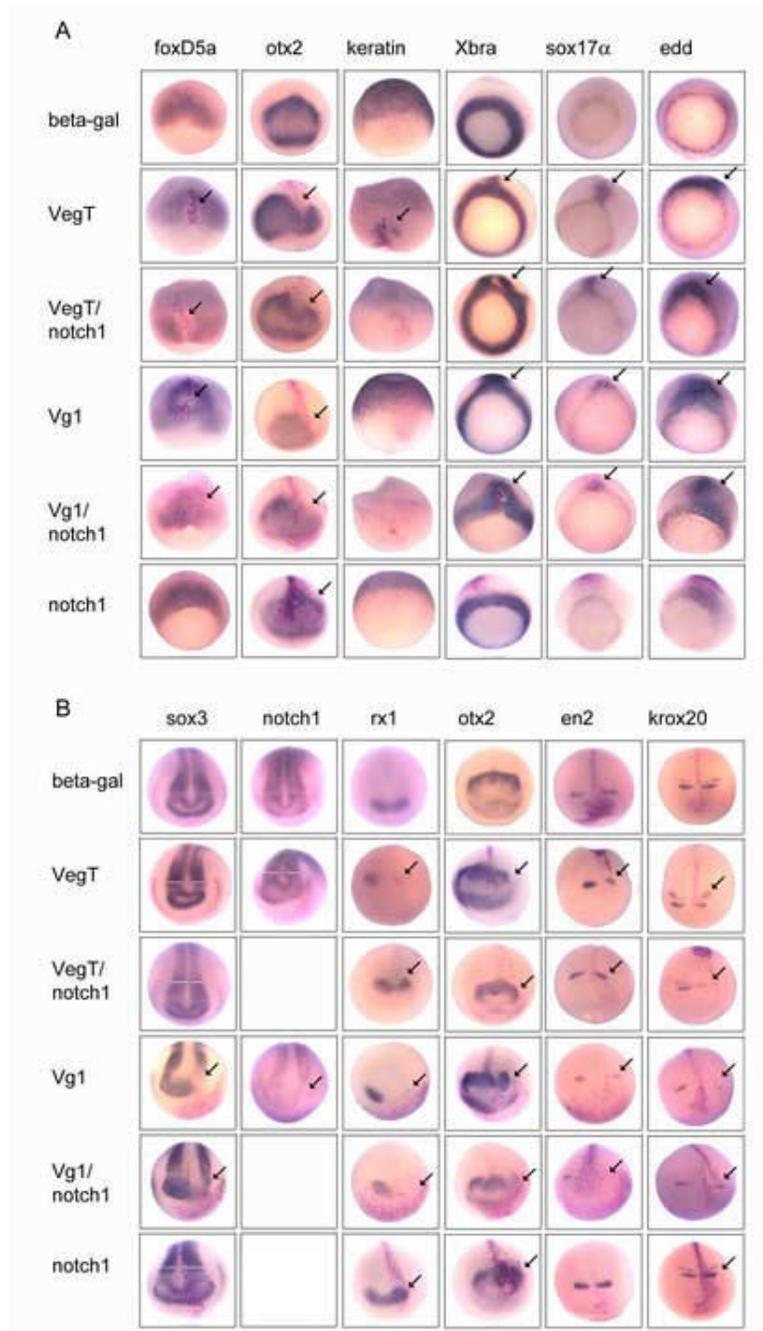
injection of *notch1* did not increase the retinal size of *Vg1*-injected embryos compared to *Vg1* alone. Co-injection of *VegT* and *Vg1* mRNAs further reduced the size of the retina compared to *VegT* alone ($p < 0.01$) or *Vg1* alone ($p < 0.05$).

(C) Percentage of embryos in which D1.1 progeny populate the retina. The number is dramatically reduced in a dose-dependent manner in embryos injected with *VegT* mRNA.

(D) Percentage of embryos in which D1.1 progeny populate the retina. The number is dramatically reduced in a dose-dependent manner in embryos injected with *Vg1* mRNA; this is not reversed by co-injection of *notch1* (500pg).

(E) The retina (r) on the injected (right) side of *VegT* and *Vg1* embryos is smaller, and the D1.1 progeny (green) that normally contribute large numbers to the retina and forebrain (fb) in GFP controls are missing in *VegT* and *Vg1* embryos. Scale bar = 200 μ m.

(F) The size of the D1.1 clone (green) in the gut (g) is larger in *VegT*- and *Vg1*-injected embryos compared to GFP controls. h, heart; n, notochord. Scale bar = 200 μ m

**Figure 3.**

VegT and Vg1 alter cell fates at gastrulation stages and these changes affect later neural patterning.

(A) The expression domains of markers of the germ layers (top labels) during gastrulation after injection of one D1.1 blastomere with mRNAs indicated on the left. Early neural ectoderm is identified by *foxD5* and *otx2*, non-neural ectoderm by *keratin*, mesoderm by *Xbra* and endoderm by *sox17α* and *edd*. Red cells indicate the D1.1 progeny expressing the injected mRNA, and arrows indicate regions of gene repression or ectopic expression. Frequencies of phenotypes are presented in Table 1.

(B) The expression domains of pan-neural (*sox3*, *notch1*), eye field (*rx1*), forebrain (*otx2*), midbrain (*en2*) and hindbrain (*krox20*) genes during neural plate stages. White bars indicate the width of the expression domains on the injected (right) versus uninjected (left) side of the neural plate. Arrows are as above. Frequencies of phenotypes are presented in Table 1.

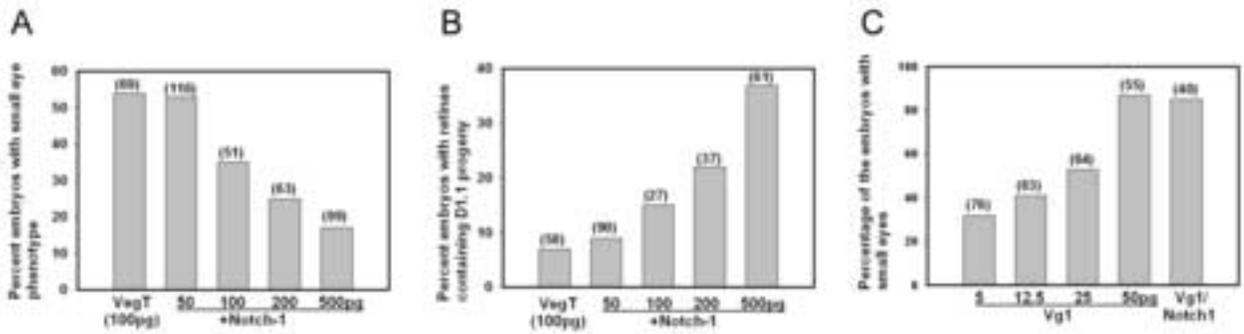


Figure 4.

Co-expression of *notch1* reverses *VegT* retinal effects but not those of *Vg1*.

(A) Co-expression of *notch1* reduces the frequency of the *VegT* small eye phenotype in a dose-dependent manner. Numbers in parentheses indicate size of sample.

(B) The frequency that *VegT*-expressing D1.1 cells populate the retina increases with increasing doses of *notch1*.

(C) *Vg1* causes a small eye phenotype in a dose-dependent manner, which is not rescued by *notch1* (500pg). (See also Figure 2D).

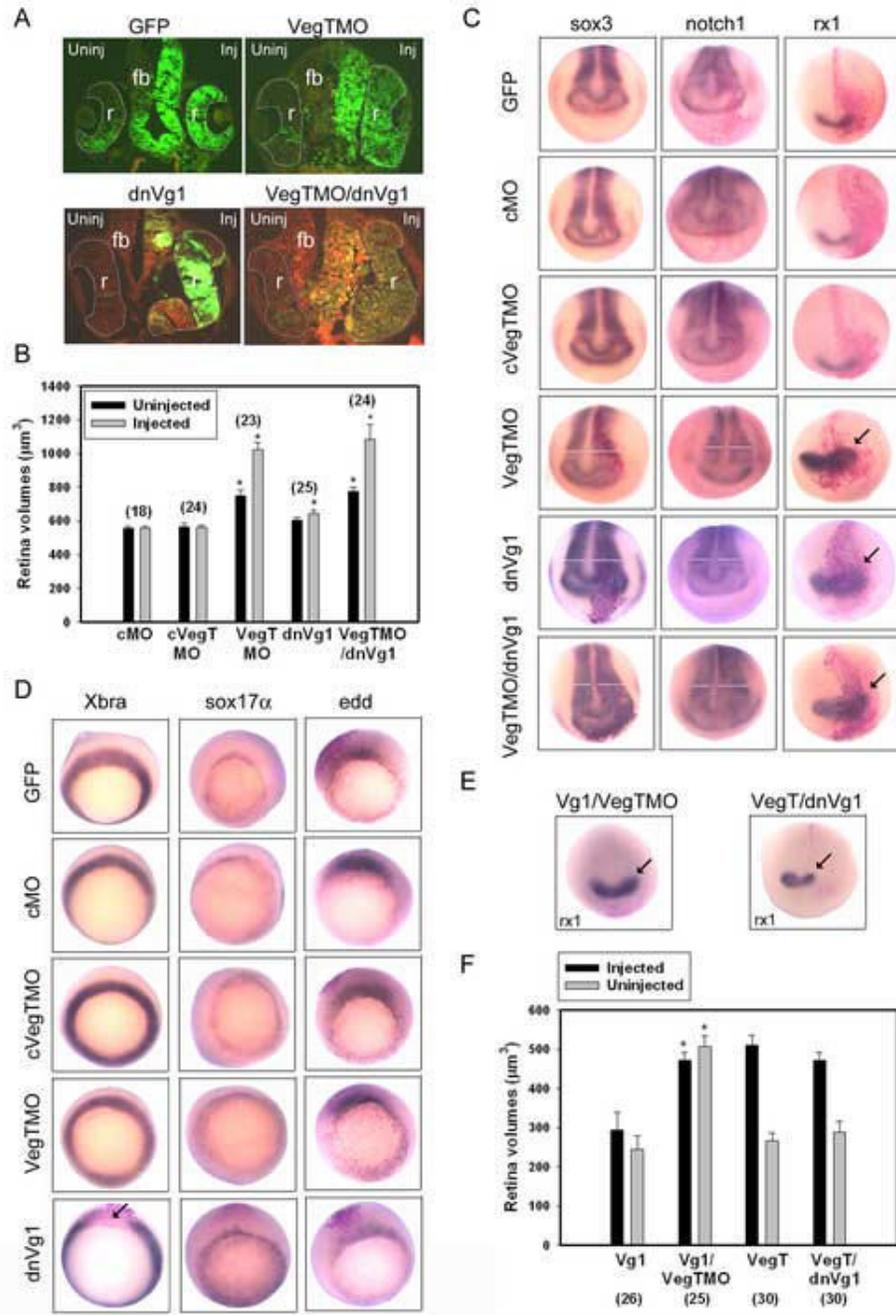


Figure 5. Reduction of VegT and Vg1 in the D1.1 lineage increases the size of the neural ectoderm. (A) Reduction of VegT by morpholino injection (VegTMO) and of Vg1 by expression of a dominant-negative construct (dnVg1) enlarged the retina (r) and forebrain (fb). Note the abundant D1.1 progeny (green) in both structures (*cf.* Fig. 2E). Reduction of both factors (VegTMO/dnVg1) resulted in a similar phenotype. (B) The mean volumes of retinas in embryos in which control morpholinos (cMO, cVegTMO) were injected are not different from GFP controls (Fig. 2B). Those from VegTMO-injected embryos are significantly larger on both sides, and those from *dnVg1*-injected embryos are significantly larger on the injected side. Those from embryos injected with both constructs

(VegTMO/dnVg1) are larger than for dnVg1 alone and similar to those from VegTMO alone. * indicates $p < 0.01$ compared to GFP controls. Numbers in parentheses indicate size of sample.

(C) The expression domains of pan-neural plate (*sox3*, *notch1*; white bars indicate measurement of width of domain) and retinal (*rx1*; arrows) genes are expanded on the side (right) injected with VegTMO or *dnVg1*. The expansion of neural plate markers is somewhat enhanced in embryos co-injected with VegTMO and dnVg1 (see also Table 1).

(D) The expression domains of mesodermal (*Xbra*) and endodermal (*sox17 α* , *edd*) genes after injection into D1.1 of the constructs indicated on the left. The only notable effect is repression of *Xbra* by *dnVg1* (arrow).

(E) The expansion of *rx1* expression (arrow) by VegTMO is not altered by co-expression of Vg1, whereas the expansion caused by *dnVg1* is reversed by co-expression of VegT.

(F) The small retinal volumes displayed by *Vg1*-injected embryos were significantly increased by co-injection of VegTMO (* indicates $p < 0.01$). The small retinal volumes displayed by VegTMO-injected embryos were not altered by co-injection of *dnVg1* ($p > 0.05$).

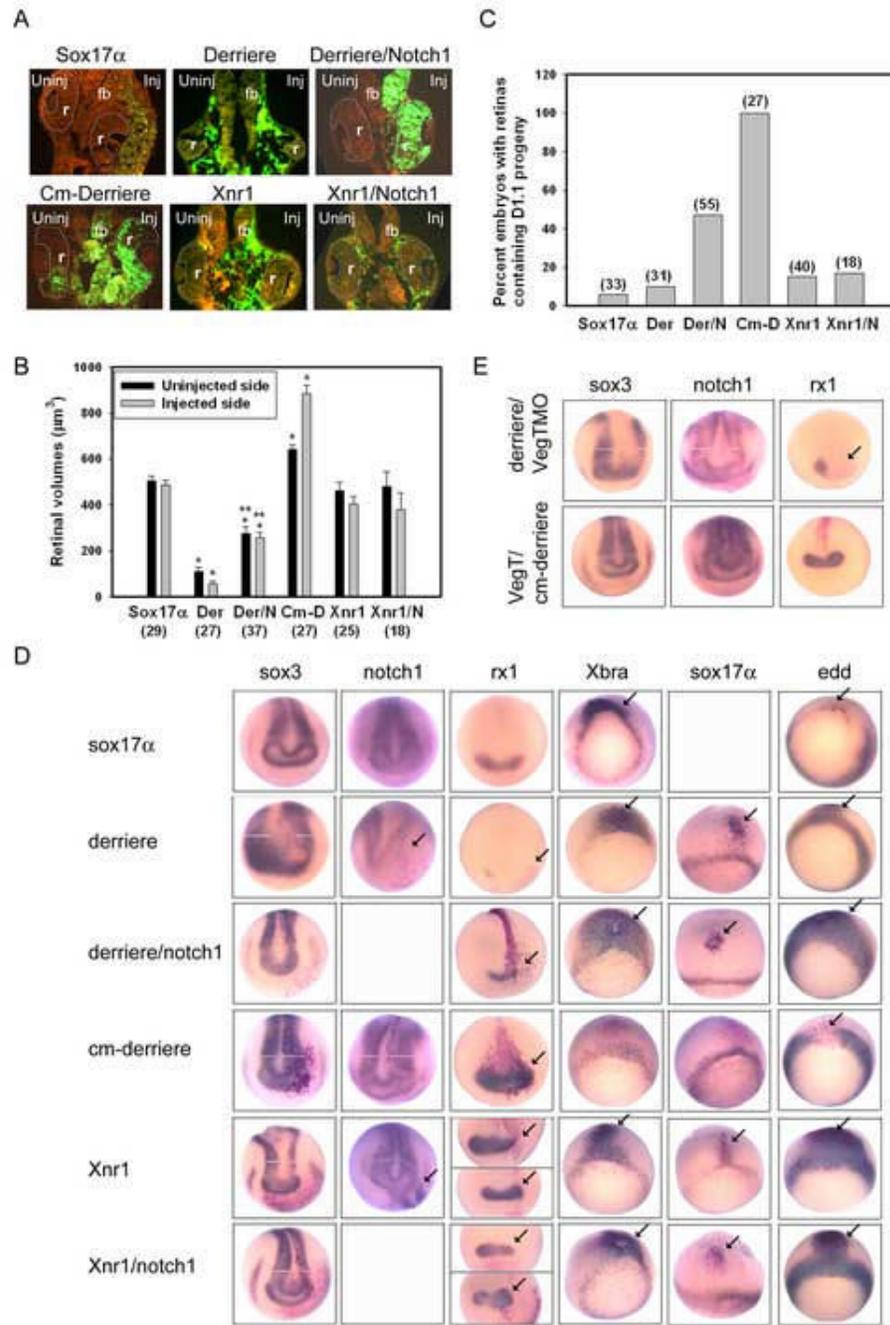


Figure 6. The combination of *sox17 α* and *derriere* phenocopies the VegT effect on the D1.1 lineage. (A) The retinas (r) in *sox17 α* and *Xnr1* mRNA injected embryos are normal in size, whereas *derriere* dramatically reduced both retinas. However, D1.1 progeny (green) were not located within the retina in *sox17 α* , *derriere* or *Xnr1* injected embryos. Co-expression of *notch1* reversed the *derriere* phenotype, resulting in large retinas populated by abundant D1.1 progeny (green), but did not alter the *Xnr1* phenotype. The expression of a dominant-negative *derriere* construct (*Cm-derriere*) resulted in a larger retina populated by large numbers of D1.1 progeny.

(B) The mean volume of retinas in embryos injected with mRNAs for *sox17a*, *derriere* (Der), *derriere* plus *Notch1* (Der/N), dominant-negative *derriere* (Cm-D), *Xnr1* and *Xnr1* plus *notch1* (*Xnr1/N*). *sox17a*- and *Xnr1*-injected embryos do not significantly differ from GFP controls (Fig. 2B), whereas those from *derriere* embryos are significantly reduced on both sides. Co-expression of *Notch1* partially rescues the *derriere* effect but has no effect on *Xnr1* retinas (*, $p < 0.01$ compared to GFP controls; **, $p < 0.05$ compared to *derriere*). Cm-D significantly increased the size of both retinas (*, $p < 0.01$ compared to GFP controls).

(C) Percentage of embryos in which D1.1 progeny populate the retina is dramatically reduced in embryos injected with *sox17a*, *derriere* (Der) or *Xnr1* mRNAs. The *derriere* phenotype is partially rescued by co-expression of *notch1* (Der/N), whereas the *Xnr1* phenotype is not (*Xnr1/N*). All Cm-D expressing embryos have D1.1 progeny in the retinas, identical to GFP controls (Fig. 2C)

(D) Domains of pan-neural plate (*sox3*, *notch1*), retinal (*rx1*), mesodermal (*Xbra*), and endodermal (*sox17a*, *edd*) genes after injection of one D1.1 blastomere with constructs listed on the left. The injected side of the embryo is on the right and the uninjected side is on the left. The sites of effects are indicated either by arrows, or in the neural plate by white bars indicating the width of the expression domains. Expression of *sox17a* does not affect neural/retinal genes, but inhibits *Xbra* and expands *edd*, consistent with its role in converting cells to an endodermal fate. Expression of *derriere* represses neural and retinal genes, and these effects are rescued by *notch1*. Cm-Derriere expanded neural and retinal markers and repressed *Xbra*. *Xnr1* either represses (*sox3*, top *rx1* panel) or expands (*notch1*; bottom *rx1* panel) neural/retinal genes, and endo-mesoderm markers are dorsally expanded. No *Xnr1* phenotype is rescued by *notch1*.

(E) The expansion of neural (*sox3*, *notch1*) and retinal (*rx1*) expression domains by injection of VegTMO (Fig. 5C) is reversed by co-injection of *derriere*. Reduction of these markers by *VegT* (Fig. 3B) is reversed by *cm-derriere*. (See also Table 1).

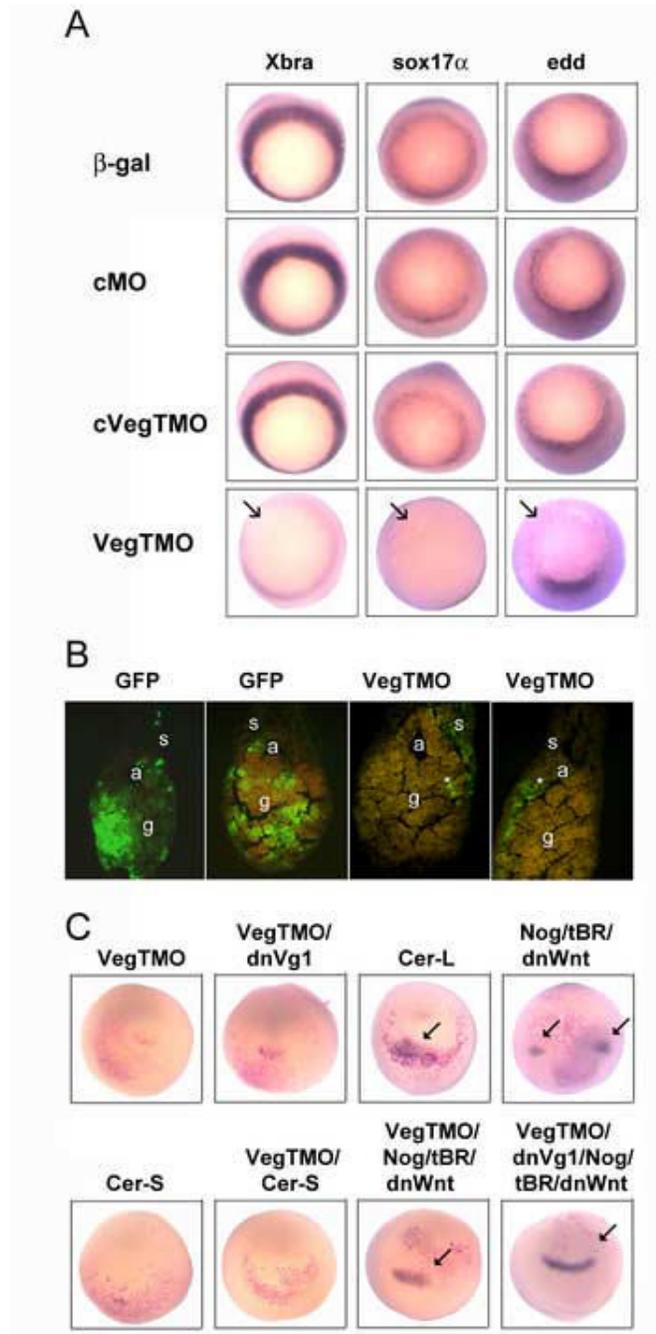


Figure 7.

In order to convert the V2.1.1 lineage to a retinal fate endo-mesodermal factors must be blocked in combination with suppression of BMP and Wnt signaling.

(A) Expression domains of mesodermal (*Xbra*) and endodermal (*sox17 α* , *edd*) genes are reduced by VegTMO injection into the vegetal pole, whereas controls (β -gal, cMO, cVegTMO) have no effects.

(B) The extent to which the hindgut (g) is populated by V2.1.1 progeny (green) is strikingly reduced in VegTMO embryos (*) compared to controls (GFP). a, archenteron; s, somite.

(C) Ectopic expression of *rxI* (blue, arrows) in the vegetal pole was monitored by in situ hybridization after the V2.1.1 blastomere was injected with the indicated constructs. Red cells

indicate the V2.1.1 progeny expressing those constructs. Reduction of VegT alone (VegTMO) or in combination with Vg1 (VegTMO/dnVg1) was not sufficient to induce ectopic *rx1* expression. The long form of Cerberus (Cer-L), which can inhibit BMP, Wnt and Nodal signaling, was effective whereas the short version of Cerberus (Cer-S), which can inhibit only Nodal signaling, was not. Although reduction of both BMP signaling (by expression of noggin [Nog] and a dominant-negative BMP receptor [tBR]) and Wnt signaling (by expression of a dominant-negative Wnt8 construct [dnWnt]) causes ectopic *rx1* expression, the cells expressing *rx1* are not derived from the V2.1.1 clone (red cells), demonstrating an indirect consequence of the induction of a secondary head (as reported in Moore and Moody, 1999). However, combining the reduction of VegT or VegT plus Vg1 with the inhibition of BMP and Wnt signaling caused the V2.1.1 clone to ectopically express *rx1*, equivalent to the Cer-L phenotype.

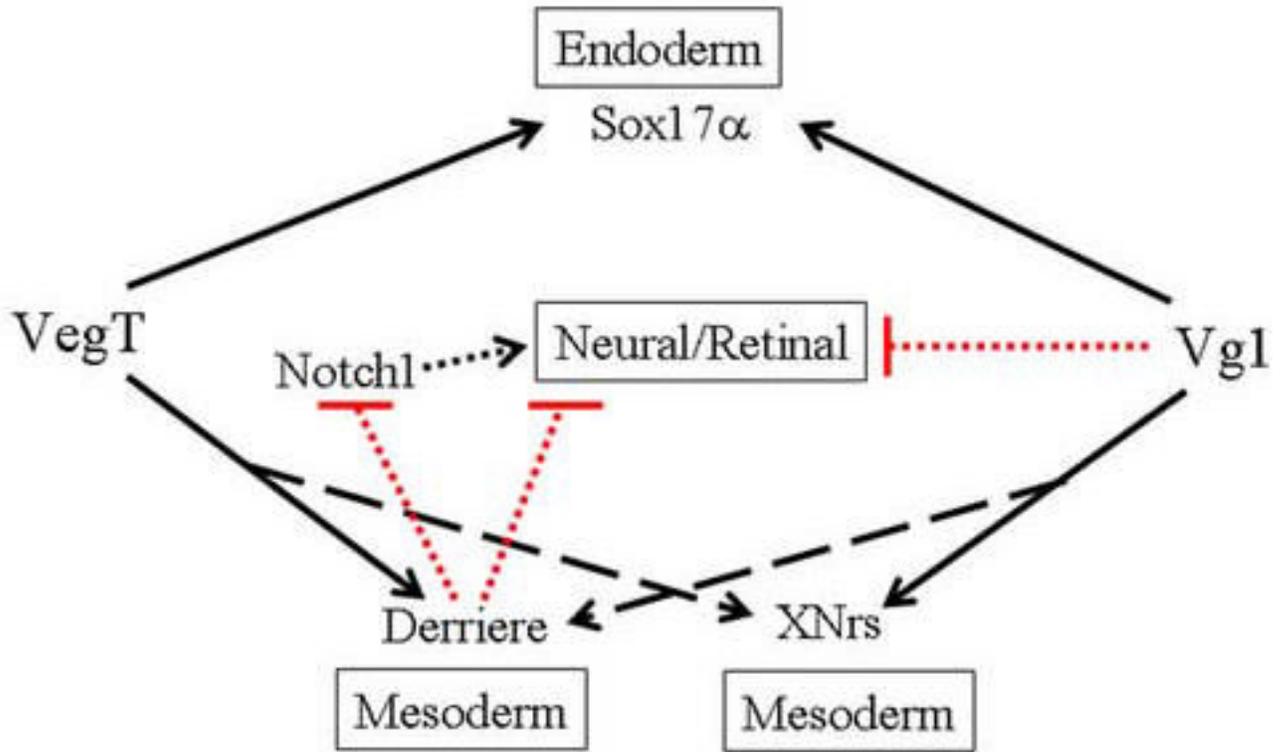


Figure 8.

VegT and Vg1 both repress the ability of a cell to express a neural/retinal fate via overlapping but not identical pathways. Both dominantly promote (black arrows) endodermal and mesodermal fates via up-regulation of Sox17 α , Derriere and XNrs (broken arrows indicate that the strength of the effect may not be equally strong for VegT and Vg1). Derriere acts downstream of VegT. Notch1, which promotes neural/retinal fates (black arrow), is proposed as an intermediary between VegT/Derriere repression of neural/retinal fate because: a) both VegT and Derriere repress the Notch1 expression domain; and b) Notch1 can rescue the repression of neural/retinal fates by both VegT and Derriere. But, Notch may not be the only pathway by which VegT/Derriere repress neural/retinal fates. Notch1 does not rescue Vg1 or XNr1 repression of neural/retinal fates. Dashed lines indicate that the effects may not be direct.

Table 2

The ventral vegetal lineage expresses a retinal fate only when vegetal factors, anti-neural factors and posterior-axis promoting factors are inhibited

Injected mRNAs or morpholinos	ISH		Fate Mapping	
	ectopic expression (% embryos)		clone populates (% embryos)	
	<i>sox3</i> (n)	<i>rx1</i> (n)	neural (n)	retina (n)
β -gal	0 (23)	0 (31)	-	-
gfp	-	-	0 (23)	0 (23)
VegTMO	0 (32)	0 (32)	30 (27)	0 (27)
dnVg1	0 (24)	0 (26)	32 (25)	0 (25)
VegTMO/dnVg1	0 (32)	0 (34)	33 (18)	0 (18)
cer-L	38 (32)	33 (48)	80 (30)	27 (30)
nog/tBR/dnWnt	41 (32)	32 (38)	79 (19)	0 (19)
cer-S	-	0 (37)	-	0 (31)
cm-Xnr2	-	0 (46)	-	0 (42)
cer-S/cm-Xnr2	-	0 (38)	-	0 (34)
cm-derriere	-	0 (30)	-	0 (27)
VegTMO/cer-S	-	0 (26)	-	0 (34)
VegTMO/cm-Xnr2	-	0 (38)	-	0 (28)
VegTMO/cm-derriere	-	0 (34)	-	0 (22)
VegTMO/nog/tBR/dnWnt	59 (32)	37 (38)	50 (29)	38 (26)
VegTMO/tBR	-	0 (26)	-	0 (34)
VegTMO/dnWnt	-	0 (31)	-	0 (27)
VegTMO/dnVg1/nog/tBR/dnWnt	100 (30)	100 (26)	100 (6)	45 (29)

Legend: Reagents were injected into one V2.1.1 blastomere with appropriate lineage tracer (β -Gal for ISH; GFP for fate mapping). Embryos were fixed at neural plate stages for ISH and stages 37/38 for fate mapping.