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Regulation of programmed cell death during neural induction in the chick embryo

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ABSTRACT To study early responses to neural inducing signals from the organizer (Hensen's node), a differential screen was performed in primitive streak stage chick embryos, comparing cells that had or had not been exposed to a node graft for 5 hours. Three of the genes isolated have been implicated in Programmed Cell Death (PCD): Defender Against Cell Death *(Dad1)*, Polyubiquitin II *(UbII)* and Ferritin Heavy chain *(fth1)*. We therefore explored the potential involvement of PCD in neural induction. *Dad1*, *UbII* and *fth1* are expressed in partly overlapping domains during early neural plate development, along with the pro-apoptotic gene *Cas9* and the death effector *Cas3*. *Dad1* and *UbII* are induced by a node graft within 3 hours. TUNEL staining revealed that PCD is initially random, but both during normal development and following neural induction by a grafted node, it becomes concentrated at the border of the forming neural plate and anterior non-neural ectoderm and downregulated from the neural plate itself. PCD was observed in regions of Caspase expression that are free from *Dad1*, consistent with the known anti-apoptotic role of *Dad1*. However, gain- and loss-of-function of any of these genes had no detectable effect on cell identity or on neural plate development. This study reveals that early development of the neural plate is accompanied by induction of putative pro- and anti-apoptotic genes in distinct domains. We suggest that the neural plate is protected against apoptosis, confining cell death to its border and adjacent non-neural ectoderm.

KEY WORDS: *dad, ubiquitin, PCD, apoptosis, neural plate, neural ectoderm*

Introduction

Neural induction is the process by which signals emanating from cells in the "organizer" (Hensen's node in amniote embryos) instruct cells in the adjacent epiblast to acquire a neural fate and form the neural plate, from which the entire Central Nervous System (CNS) arises (Stern, 2005). Several lines of evidence implicate BMP inhibition as an essential step in neural induction (de Almeida *et al.*, 2008, Harland, 2000, Hemmati-Brivanlou and Melton, 1997, Linker *et al.*, 2009, Linker and Stern, 2004, Marchal *et al.*, 2009, Reversade *et al.*, 2005, Stern, 2006, Vonica and Brivanlou, 2006). However chick epiblast cells cannot respond to BMP inhibitors unless they have previously been exposed for at least 5 hours to other signals from the organizer (Linker and Stern, 2004, Streit *et al.*, 1998).

To define this essential early step at the molecular level, we designed a differential screen between cells that either had or had not been exposed to a graft of Hensen's node for 5 hours (Streit *et al.*, 2000). Among the genes isolated were several previously undescribed genes such as *ERNI* (Papanayotou *et al.*, 2008, Streit *et al.*, 2000) and *Churchill* (Sheng *et al.*, 2003). Three of the genes correspond to the chicken homologues of known genes: Defender Against Cell Death 1 (*Dad1*), Polyubiquitin 2 (*UbII*) and Heavy chain Ferritin (*fth1*). Their products have been implicated in controlling cell death in other systems, raising the possibility

Abbreviations used in this paper: CNS, Central Nervous System; Dad, defender against cell death; fth, ferritin heavy chain; PCD, programmed cell death; Ub, ubiquitin

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that these genes, and regulation of apoptosis, may play a role at early stages of neural development.

Here we describe their expression patterns and the distribution of apoptotic cells during neural induction in the chick. Gain- and loss-of-function experiments suggest that these genes are not essential for neural plate formation. However, regulation of the spatial distribution of programmed cell death accompanies the early stages of neural induction along with the induction of expression of these genes. We suggest that the early neural plate is protected against apoptosis, restricting cell death to the border of the neural plate (from which the neural crest and placodes will eventually arise) and the adjacent anterior non-neural ectoderm.

Results

Expression of Dad1, UbII *and* Fth1 *during neurulation*

Weak expression of *Dad1* is first detected at mid-primtive streak stages (about stage 3), in a region corresponding approximately to the prospective neural plate (Fig. 1A), which then intensifies (Fig. 1 B, E). During neurulation and early somite stages, *Dad1* is expressed predominantly in the forming neural plate, especially in the prospective neural crest (Fig. 1 D, F, arrows in F) and more weakly in somites and the regressing primitive streak (Fig. 1 C, D, F).

UbII is expressed in the anterior half of the epiblast at primitive streak stages, including the prospective neural plate and prospective neural plate border (Fig. 1 G). It then becomes concentrated at the anterior neural border (Fig. 1 H, I, arrow in K) and later to the neural tube (Fig. 1 J, L) and migrating neural crest (arrowheads in Fig. 1J).

The expression of *fth1* is quite variable at early stages: at stages 3^{+}-5 some embryos show fairly uniform staining throughout the area pellucida (Fig. 1M), others show expression concentrated in non-neural regions (excluding the presumptive neural plate; Fig. 1N) and yet others have expression in the future neural plate but not prospective epidermis (Fig. 1O). The pattern becomes consistent after stage 7, when the strongest expression is seen in developing blood islands and the developing neural plate (Fig. 1 P-R).

In conclusion, all three genes isolated from the differential screen for early responses to neural induction are expressed in the neural plate of normal embryos from late primitive streak to early neurulation stages. However they differ in their precise patterns and timing of expression.

Gain- or loss-of-function of Dad1, UbII *or* Fth1 *has no effect on cell identity*

To assess whether these three genes influence cell fate choices at early stages of neural development, they were variously either misexpressed by electroporation and/or their function abrogated using dominant-negative constructs or morpholinos. Neither gain- nor loss-of-function of any of these genes had any effect on neural, epidermal or neural border marker expression (for details see Supplementary Information).

Localization of cells undergoing apoptosis during normal development

Dad1, UbII and fth1 have all been implicated in the regulation of cell death (Cairo *et al.*, 1995, Hong *et al.*, 2000, Lee and Peter,

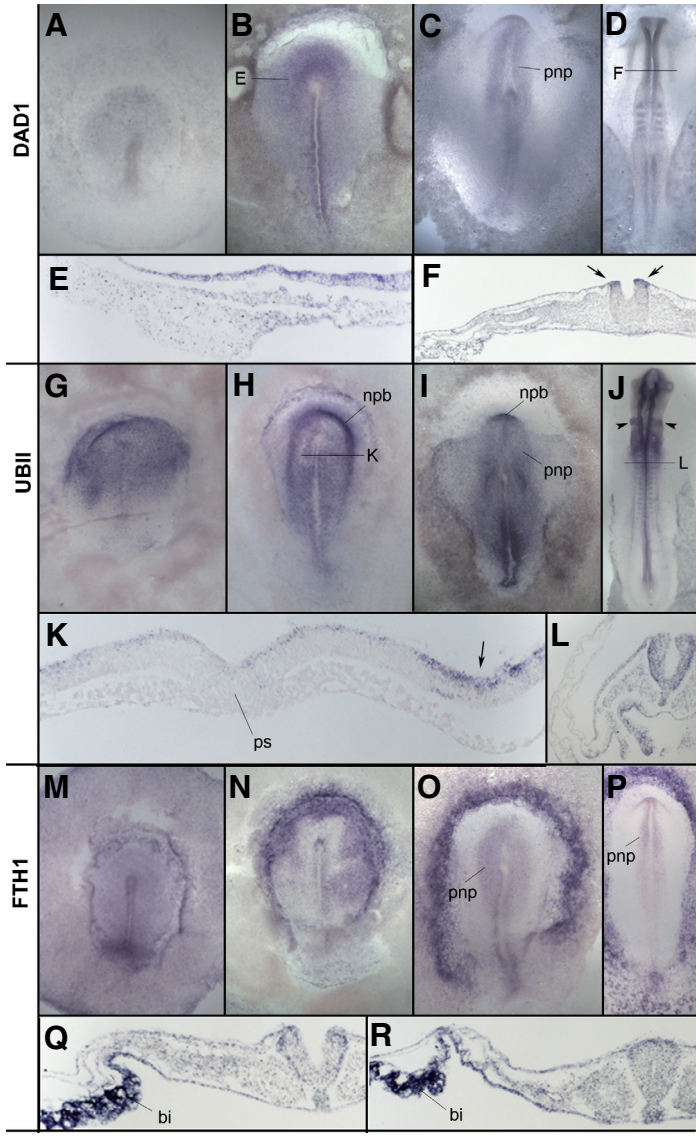

Fig. 1. Expression of *Dad1, UbII* and *Fth1*. (A-F) *Expression of* Dad1 *at stages 3* **(A)**, *4* **(B)**, *6* **(C)** *and 8* **(D)** *showing progressive restriction to the forming neural plate. Histological sections (E, F, at the levels indicated in panels B,D) show that early expression is concentrated in the epiblast layer (E) and that during neural plate formation expression is strongest at the neural plate border, in prospective neural crest (F, arrows).* **(G-L)** *Expression of* UbII. *At early stages expression is seen in the prospective neural plate (G; stage 3^{+}), then becomes concentrated at its border (H; stage 5 and I, stage 6) and eventually throughout the entire nervous system including the migrating neural crest (arrowheads in J; stage 11). Transverse sections through embryos at stage 5 show expression concentrated in the neural/epidermal border region (K, arrow) and increased expression in the neural tube at stage 11 (L).* **(M-R)** *Expression of Fth1 is initially ubiquitous (M; stage 4^{-}). Considerable variation is seen at stage 4, with some embryos expressing mainly outside the neural plate (N) and others with low expression in non-neural ectoderm, higher in the neural plate and higher still in extraembryonic tissues (O; stage 5^{-}). By stages 6-7 expression is seen more consistently in the neural plate (P). At later stages (Q,R, transverse sections through a stage 11 embryo), expression in the neural plate decreases slightly whereas the extraembryonic blood islands (bi) express very strongly. pnp, prospective neural plate; npb, neural plate border; ps, primitive streak; bi, blood islands.*

2003, Nakashima *et al.*, 1993, Yang *et al.*, 2002). We therefore used TUNEL staining to explore the distribution of apoptosis during normal development and in response to neural induction by a grafted node. TUNEL staining appears random until stage 4 (Fig. 2 A-G). From stage 4⁺-5 (Fig. 2 H-K) apoptotic cells start to concentrate in an arc parallel to the anterior border of the neural plate, extending latero-caudally. As the embryo elongates and narrows (stages 5-14), the arc expands caudally, reminiscent of the early patterns of expression of *BMP4* and *BMP7* (Streit *et al.*, 1998, Streit and Stern, 1999). From stages 8-9, TUNEL-positive cells are seen in the remnants of the primitive streak at the caudal tip of the axis (Fig. 2 L-N). The dorsal midline of the neural tube shows an increase in the number of TUNEL positive cells in regions where the neural folds are fusing (Fig. 2 M-P). In addition, there are two regions of concentrated apoptosis, one in the rhombencephalon (Fig. 2 M-P) which eventually becomes restricted to rhombomeres 3 and 5 (Graham *et al.*, 1993, Jeffs *et al.*, 1992) and another in the olfactory region at stages 9 -14 (Fig. 2 N-Q; see also Yang *et al.* (1998). At stage 13, apoptotic cells are seen at the edge of the anterior intestinal portal (Fig. 2Q) and anterior epidermis (Fig. 2 P, Q).

Hensey and Gautier also observed an arc of dying cells in *Xenopus* embryos at neurula stages and interpreted it as being within the neural plate (Hensey and Gautier, 1998). To determine more precisely the position of the arc in chick embryos, TUNEL staining was combined with *in situ* hybridization for neural plate

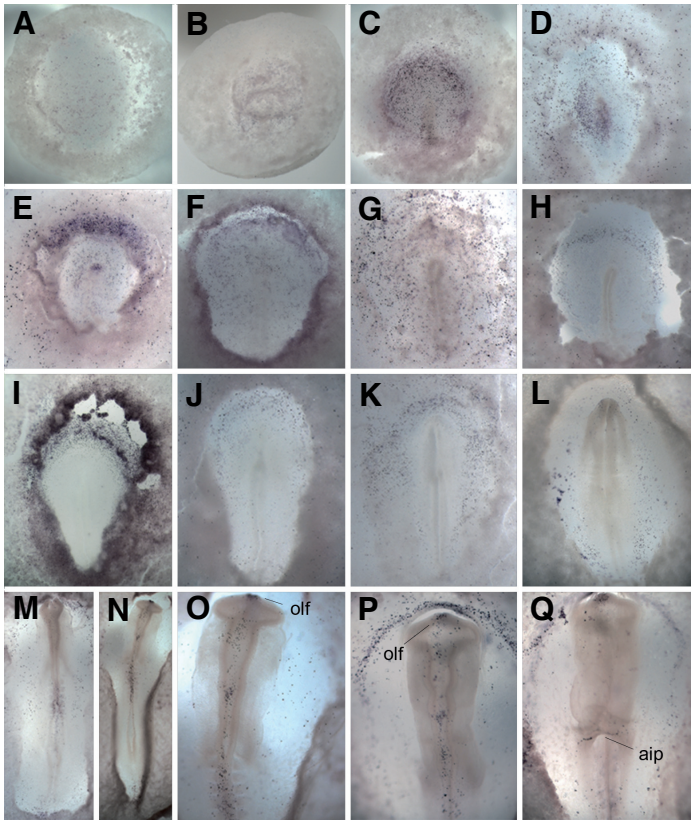

Fig. 2 (Left). Distribution of apoptosis revealed by TUNEL staining. **(A-G)** *At early stages, TUNEL-positive cells are seen randomly scattered throughout the embryo (A, stage XII; B, stage XIII; C, stage 2⁺; D, stage 3; E, stage 3⁺; F, stage 4⁻; G, stage 4).* **(H-L)** *From stage 4, PCD is more consistently concentrated in an arc at the neural/non-neural border and adjacent anterior non-neural ectoderm (H, stage 4; I, stage 4⁺; J, stage 5⁻ ; K, stage 5). In some embryos the arc of apoptotic cells extends far caudally at stages 6-8 (K and L, stage 8).* **(M-Q)** *By early somite stages (M, stage 8+; N, stage 9, O, stage 10, P, Q stage 11 with P viewed from dorsal side and Q from ventral side). PCD is seen in the olfactory region (olf) at the anterior tip of the neural tube (N-Q), fusing neural folds (M-P) especially in the rhombencephalon and in the anterior intestinal portal endodermal region (Q). The arc of anterior epidermal dying cells is still seen at these stages (P). olf, olfactory region; aip, anterior intestinal portal.*

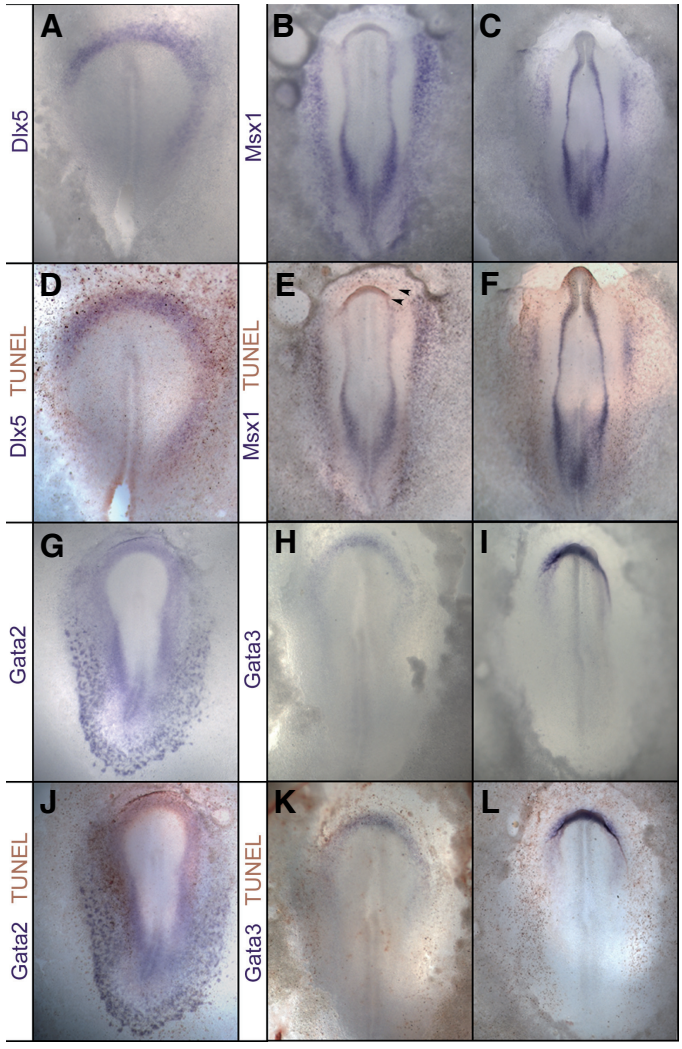

Fig. 3 (Right). Comparison of regions of cell death with neural border and epidermal markers. *TUNEL staining overlaps the region expressing the border markers* Dlx5 *(A, D; stage 4⁺) and* Msx1 *(B, C, E, F; stage 8). However the region of PCD also enters the prospective epidermis marked by* Gata2 *(G,J; stage 5) and* Gata3 *(H,I,K,L; stage 6). Each embryo is shown after initial* in situ *hybridization for the marker indicated, in blue (A-C, G-I) and following TUNEL staining after in situ, in brown (D-F, J-L). In (E), note the double arc of TUNEL staining (arrowheads). An inner arc overlaps with Msx1 staining at the border of the neural plate, and there is also a more peripheral, epidermal arc.*

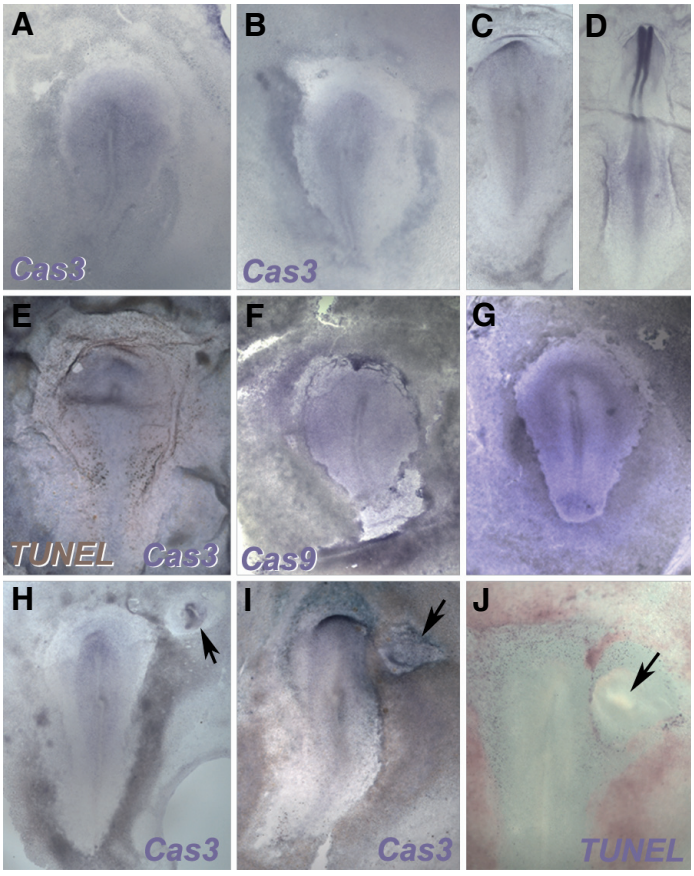

Fig. 4 (Above). Expression of caspases and responses to neural induction. (A-E) *Expression of* Caspase3 *at stage 4⁺ (A), 5 (B), 6 (C) and 8 (D). Panel (E) shows an embryo at stage 4⁻ double-stained for* Caspase3 *(blue) and TUNEL (brown): apoptotic cells lie just beyond the edge of the* Cas3*-expressing domain, in the non-neural ectoderm.* **(F,G)** *Expression of* Caspase9 *is widespread at stage 4⁺ (F) but starts to clear from the neural plate and becomes concentrated at its border at stage 5 (G).* **(H-J)** *Responses to neural induction by a grafted node (arrows). The graft induces an initial domain of* Caspase3 *expression (H,I) similar to the pattern in the normal neural axis. TUNEL staining shows that the induced neural plate is devoid of apoptosis, which becomes concentrated as a sharp line between the induced and host neural plate (J).*

markers (*Sox3* and *Sox2*; not shown), neural/epidermal border markers *Dlx5* and *Msx1* (prospective neural crest and pre-placodal regions; (McLarren *et al.*, 2003) and early epidermal markers *GATA2* and *GATA3* (Sheng and Stern, 1999) (Fig. 3). Apoptotic cells can be seen both outside and inside of the neural-epidermal border, but at stage 5 the pattern is more concentrated in a region overlapping with the expression of the border markers *Msx1* and *Dlx5* (Fig. 3 A-F) and extending into the epidermal (*Gata2* and *Gata3*) domain (Fig. 3 G-L). Some embryos have two parallel arcs, one at the border of the neural plate and a second in the non-neural epidermis (e.g. Fig. 3E, arrowheads). Around stage 6-8, the arc at the border of the neural plate starts to disappear but the more distant arc in the non-neural ectoderm remains. As the neural plate elevates and starts to close (stages 8-10), the arc of PCD remains in the non-neural ectoderm (Fig. 2 P, Q) whereas PCD at the neural plate border becomes confined to sub-regions of the neural folds (Fig. 2 M-P) and prospective olfactory region (Fig. 2 N-Q). In conclusion, cell death is concentrated at the neural-epidermal border and proximal non-neural epidermis, especially in the head.

Expression of Cas3 *and* Cas9 *during early development*

Apoptotic death is controlled by the activity of specific caspases. To determine whether the localization of TUNEL staining corresponds to areas of high caspase expression, we first examined the expression of *Caspase 3* (*Cas3*), the most downstream caspase in the cascade (Assefa *et al.*, 2004, Faleiro *et al.*, 1997, Li *et al.*, 1997). No specific expression could be detected in any region before stage 3 (not shown). From stage 4, *Cas3* starts to be expressed weakly in the central epiblast, primitive streak and early neural plate (Fig. 4 A-C) and eventually in the forming neural tube (Fig. 4D). This pattern differs from TUNEL staining at these stages, which is concentrated at the neural/epidermal border and epidermis (see above): double-staining for TUNEL and *Cas3* reveals apoptosis abutting the edge of the *Cas3*-expressing domain (Fig. 4E).

Expression of the more upstream caspase *Cas9* was also examined (Srinivasula *et al.*, 1998). It also starts weak and ubiquitous (Fig. 4F), but from stage 5 a region of slightly upregulated expression is seen surrounding the neural plate (Fig. 4G), not unlike the domain revealed by TUNEL staining

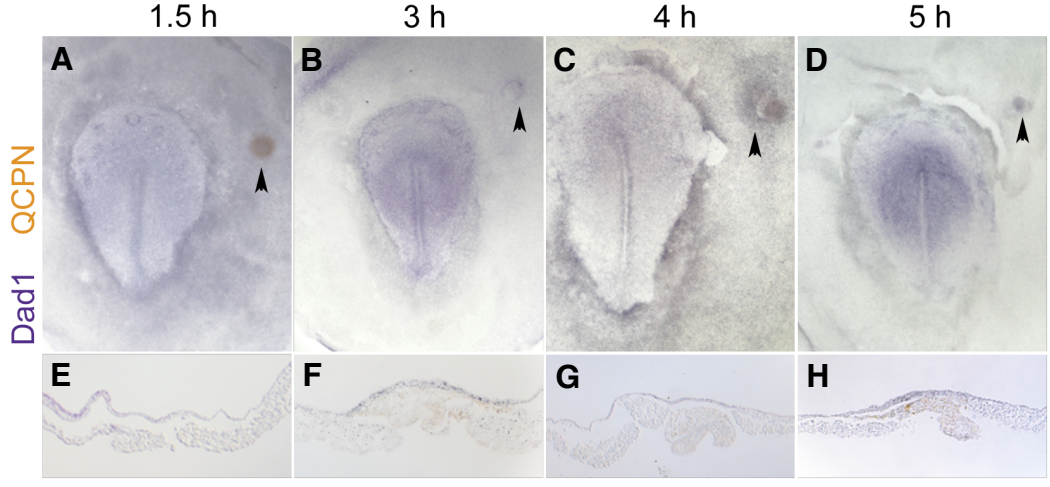

Fig. 5. Time course of induction of *Dad1* by a graft of Hensen's node. *Grafts of quail Hensen's nodes (arrowhead) into chick hosts (both donor and host at stage 3⁺) were analyzed at different time points following the transplant. No induction is seen after 1.5 hours (A,E), weak induction begins at 3 hours (B,F) and becomes more robust by 4-5 hours.* **(E,F)** *Sections through the grafted region of the embryos in (A-D), respectively, following* in situ *hybridization for* Dad1 *(purple) and staining with QCPN antibody to reveal the quail cells in the graft (brown).*

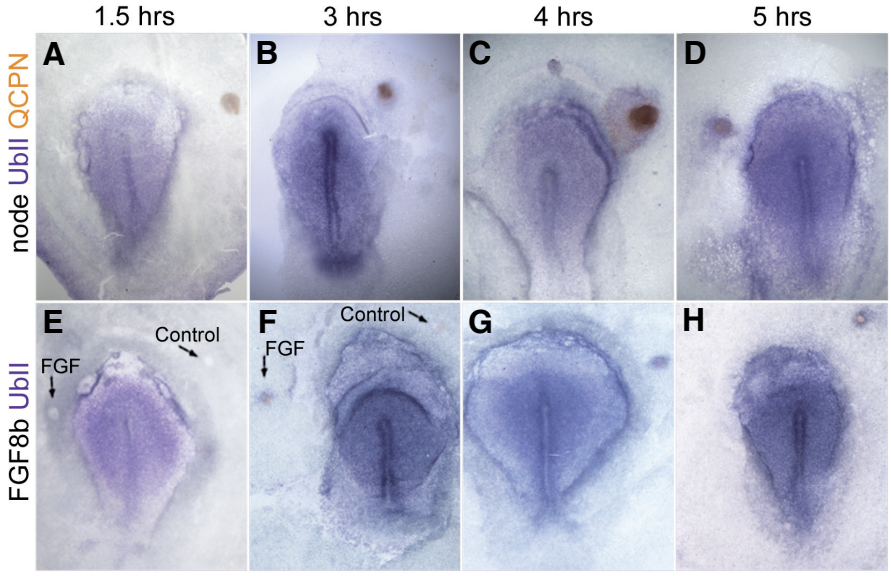

Fig. 6. Induction of *UbII* by Hensen's node and by FGF8. *Grafts of quail Hensen's nodes into chick hosts were analyzed at different time points following transplantation (A-D). Induction begins weakly at 3 hours and intensifies by 4-5 hours. A similar time-course of induction of UbII is seen following implants of FGF8-soaked beads (E-H).*

(Fig. 3). Thus, expression of *Cas9* appears to correlate more closely with TUNEL staining than does *Cas3*.

Neural induction is accompanied by downregulation of PCD

Does neural induction by the organizer regulate PCD and Caspase expression? After overnight incubation following a node graft, both TUNEL staining and *Cas3* expression in the induced epiblast mirror those in the normal embryo (Fig. 4 H-J). When the induced region is close to or overlaps with the arc of TUNEL staining of the normal embryo, apoptosis sharply decreases in the induced side. In some cases the TUNEL-positive domain is displaced away from the induced neural plate, forming a sharp border between the induced region and the host embryo (Fig. 4J). Despite some variability between embryos, the absence of apoptosis from the area surrounding the grafted node, with PCD becoming confined to the border of the induced neural plate and neighbouring epidermis, are consistent features of neural induction.

Regulation of* Dad1 *and* UbII *expression during neural induction

The differential screen pointing to these genes was designed to identify factors induced within 5 hours of receiving signals from the organizer, Hensen's node. To determine how quickly this occurs, we analyzed the expression of *Dad1* and *UbII* at different times following a node graft. *Dad1* is faintly detectable in epiblast apposed to the node graft 3 hours after the transplant and induction becomes robust by 4 hours (Fig. 5). *UbII* starts to be detected about 4 hours after grafting (Fig. 6 A-D). Induction of *UbII* can be mimicked by a graft of FGF8-soaked heparin bead (Fig. 6 E-H). We did not test the time course of induction of *Fth* because of its low level of expression in the extraembryonic epiblast.

Effects of DAD1, UBII and FTH1 on PCD

DAD1 was initially isolated as an antagonist of cell death (Nakashima *et al.*, 1993). Its expression in the forming neural plate, where it overlaps with *Cas-3*, suggests that it could function to prevent caspase-mediated cell death in this region. Consistent with this, *Dad1* expression is complementary to TUNEL staining (Fig. 7 A-D). To determine whether *Dad1* can limit cell death we compared electroporation of GFP, full-length

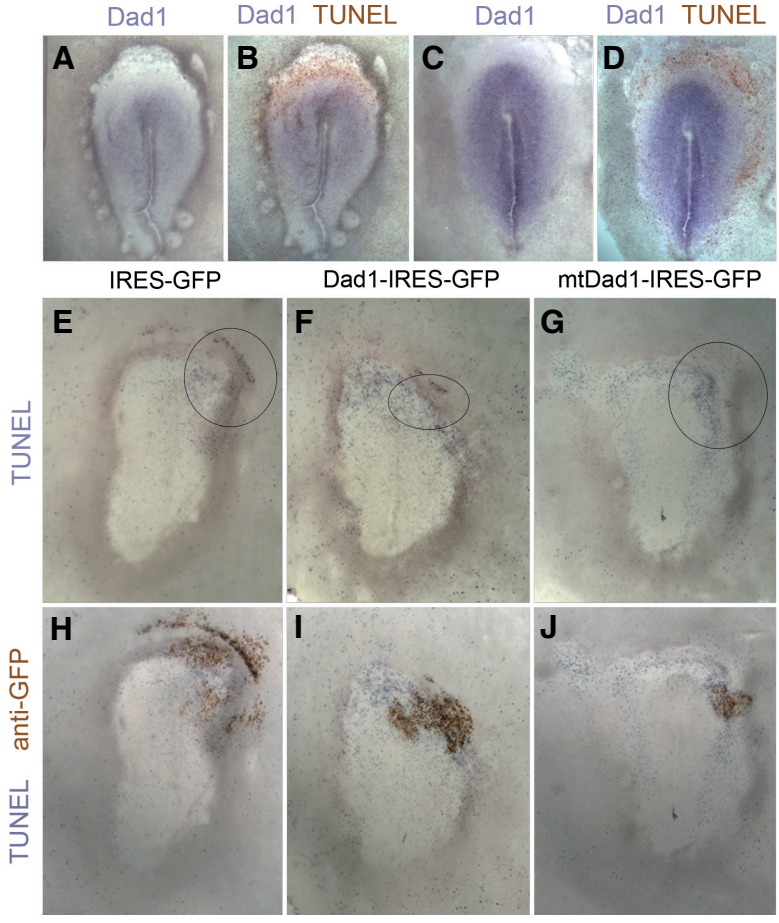

Fig. 7. Dad1 and apoptosis. (A-D) *Comparison of* Dad1 *expression with TUNEL staining shows that apoptosis is concentrated to regions of the epiblast that are comparatively free of* Dad1 *expression at stages 4 (A,B) and 5 (C,D).* **(E-J)** *Dad1 weakly inhibits apoptosis. Embryos electroporated with either empty vector IRES-GFP (E,H) or a mutated, inactive Dad1 construct (G,J) show increased apoptosis (blue in E-G) around the electroporated region (circled in E-G, then revealed by anti-GFP staining in brown in H-J). Embryos electroporated with wild-type Dad1 show a lower level of induced apoptosis in the electroporated non-neural ectoderm and neural plate border (F,I).*

Dad1 and a truncated inhibitory form of DAD1 (Makishima *et al.*, 2000). Electroporation itself slightly increases the incidence of apoptosis around the electroporated region. In some embryos (5/7 after 6 hours, 3/4 after overnight incubation) we observed a reduction of this induced apoptosis in *Dad1*-electroporated regions close to the border of the neural plate (e.g. Fig. 7 F, I), as compared to vector alone (Fig. 7 E, H) or mutated-*Dad1* (Fig. 7 G, J).

To test the role of FTH1 in apoptosis, we electroporated either full length *fth1* or a construct lacking the iron regulatory sequence (IRE) into primitive streak stage embryos. While no effect is observed 6 hours after electroporation (Fig. 8 A, D), after overnight culture the number of TUNEL-positive cells increases in embryos electroporated with *Fth* containing (in 9/10 embryos; Fig. 8 C, F) and more strongly lacking the IRE (11/11; Fig. 8 B, E). This effect is only detected in the ectoderm of the embryo proper and especially in the neuroectoderm, consistent with the idea that cell death is controlled by different mechanisms in different domains of the epiblast. This effect is not entirely cell autonomous, as it also affects some cells adjacent to those that received the construct during electroporation. No effect of *UbII* was detected on the pattern or incidence of PCD (not shown).

Together, our results suggest that the early stages of neural induction are accompanied by regulation of PCD. Induction of an ectopic neural plate by a graft of Hensen's node is accompanied by an overall inhibition of cell death in the forming neural plate. The anti-apoptotic gene *Dad1* is expressed in the nascent neural plate and induced by a graft of the organizer within 3 hours (as is

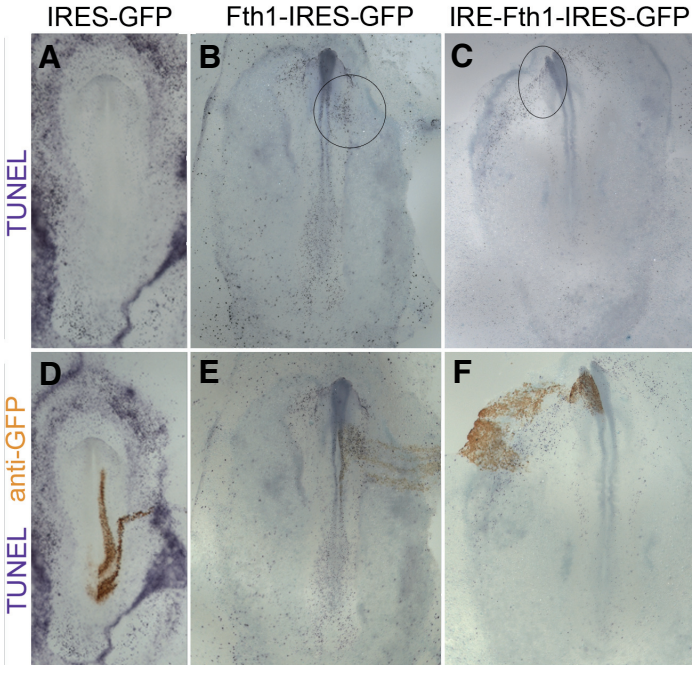

Fig. 8. Fth1 promotes apoptosis. *Embryos electroporated with* Fth1 *containing (C,F) or lacking (B,E) its iron-regulatory element (IRE) show increased apoptosis in the electroporated region as compared to embryos electroporated with the empty vector IRES-GFP (A,D). The same embryos are shown after initial TUNEL staining (in blue in A-C) and after subsequent staining for the electroporated cells with anti-GFP, (brown in D-F).*

UbII), and *Caspase3* and *Caspase9* are expressed in partly overlapping domains, with a border in the region where PCD becomes concentrated (the edge of the neural plate and adjacent epidermis). However *Dad1*, *UbII* and *Fth* appear to be dispensable during early neural plate development.

Discussion

Dad1 as anti-apoptotic factor

Defender Against Cell Death (*Dad1*) was first identified as a gene responsible for apoptotic cell death in temperature-sensitive mutants (*tsBN7*) of the hamster BHK21 cell line (Nakashima *et al.*, 1993). Since then, homologues have been identified in many species including human (Nakashima *et al.*, 1993), mouse (Makishima *et al.*, 1997), *Xenopus* and nematode (Sugimoto *et al.*, 1995), *Arabidopsis* and rice (Gallois *et al.*, 1997, Makishima *et al.*, 1997), all of which can rescue the *tsBN7* mutation. DAD1 is an effective anti-apoptotic protein but cannot protect against all apoptotic events: ectopic expression of human or nematode DAD1 prevents over 20% of PCD that would otherwise take place during normal embryogenesis in *C. elegans* (Sugimoto *et al.*, 1995).

Dad1 encodes the μ-subunit of the oligosaccharyltransferase complex OST (Kelleher and Gilmore, 1997, Makishima *et al.*, 1997, Silberstein *et al.*, 1995) but the exact mechanism by which it regulates apoptosis is not known. DAD1 binds to the anti-apoptotic protein MCL-1; however a mutant version still able to interact in this way but lacking the C-terminal 4 amino acids disrupts N-linked glycosylation and prevents rescue of the *tsBN7* mutation (Makishima *et al.*, 2000, Yoshimi *et al.*, 2000); fusion of GFP at the C-terminus also disrupts DAD1 function (Nikonov *et al.*, 2002).

Apart from the finding that DAD1 mutant mice die from excess apoptosis in the ectoderm and the distal tip of the primitive streak (Hong *et al.*, 2000), there has been no indication for a role of DAD1 in early development. Here we report that *Dad1* expression is induced as a very early response to signals from the organizer, Hensen's node, during neural induction. Its normal expression in the neural plate of the early embryo corresponds to a region where the incidence of apoptosis is particularly low. As the neural plate arises, TUNEL-positive cells accumulate at the edge of the Dad1-expressing domain, forming an arc at the border of the neural plate (future neural crest and placode area) and proximal non-neural ectoderm. These regions retain a higher incidence of apoptosis at later stages of development (Graham *et al.*, 1993, Hensey and Gautier, 1998, Jeffs *et al.*, 1992, Sanders *et al.*, 1997a, Sanders *et al.*, 1997b, Wride *et al.*, 1994). In our experiments, misexpression of DAD1 at the edges of the neural plate causes a small reduction in the incidence of PCD compared to electroporated controls in some embryos, but this is quite variable. Consistent with this variability, we did not detect any effect of loss-of-function (using either a dominant-negative version of Dad1 or antisense morpholinos) on cell death (not shown). A possible explanation is that the electroporation is done too late to eliminate all DAD1 protein from the cells. This is likely because in normal embryos *Dad1* is expressed ubiquitously at low levels, and it has also been reported that 6 hours are necessary for complete turnover of intracellular DAD1 protein when its synthesis is prevented (Makishima *et al.*, 2000). This suggests that for DAD1

levels to be lowered sufficiently it may be necessary to introduce the morpholino or inhibitory construct at a much earlier stage.

UbII and apoptosis

Ubiquitin was identified as a 74 amino acid polypeptide involved in lymphocyte differentiation, with homologs in virtually all organisms including plants (Goldstein *et al.*, 1975). Ubiquitin is considered to be the most conserved protein known: for example, yeast ubiquitin differs from those of animals by only 3 amino acids (Ozkaynak *et al.*, 1984). Typically, ubiquitins are found in the genome as polyubiquitins (Hunt and Dayhoff, 1977, Ozkaynak *et al.*, 1984), encoding repeats of the 76 amino acid Ubiquitin monomer. Ubiquitins can be added to other proteins. "Ubiquitination" refers to processes leading to selective targeting and degradation by the ubiquitin-proteasome system, while "ubiquitinylation" describes non-turnover-related post-translational modifications (Emre and Berger, 2004, Haglund and Dikic, 2005, Huang and D'Andrea, 2006, Staub and Rotin, 2006).

Links between apoptosis and polyubiquitin were established in insects (Haas *et al.*, 1995, Schwartz *et al.*, 1990) and later extended to vertebrates (reviewed in Lee and Peter, 2003). Of particular interest in the context of neural induction is the discovery of tight links between caspase activity, the cell death effector IAP, ubiquitination and NF-κB signalling, an upstream regulator of BMP (Gyrd-Hansen *et al.*, 2008, Paquette *et al.*, 2010, Tokunaga *et al.*, 2009). However, we cound not detect changes in cell death after misexpression of UbII, suggesting that it is not sufficient either to induce or to inhibit PCD at these stages of development. Upregulation of *UbII* as an early response to signals from the organizer, which we detect after only 3 hours of exposure to signals from Hensen's node, may serve functions unrelated to the regulation of apoptosis, although we could also not detect any effect of UbII misexpression on expression of various cell type markers.

FTH1 and apoptosis

Iron is crucial but also potentially toxic to cells. Ferritins play a critical role in iron sequestration (Torti and Torti, 2002). Two main types of ferritins exist in animals: heavy (FTH) and light chain, encoded by different genes of common origin (Harrison and Adams, 2002, Harrison *et al.*, 1998). The chicken genome contains a single *fth1* gene (see also Stevens *et al.*, 1987). FTH1 plays an important role in the regulation of iron homeostasis (Theil, 2003): in vertebrates, the heavy chain is responsible for accelerated oxidation of toxic Fe (II) to Fe(III), which is less toxic to cells (Levi *et al.*, 1989a, Levi *et al.*, 1989b, Quintana *et al.*, 2004). Ferritin is important from early stages of development: $Fth^{(-/-)}$ mutant mice die between E3.5-E9.5 (Ferreira *et al.*, 2000).

fth1 is regulated both transcriptionally and post-transcriptionally; similar levels of mRNA can result in a 10-fold difference in protein levels (Stevens *et al.*, 1987, Zahringer *et al.*, 1975). This regulation involves interaction between a conserved 28 bp Iron Regulatory Element (IRE) sequence in the 5' UTR and Iron Regulatory Proteins (IRPs) 1 and 2 (Eisenstein, 2000, Harrell *et al.*, 1991, Kim *et al.*, 1995, Theil, 1990, Thomson *et al.*, 1999), which repress FTH1 translation in low iron conditions.

FTH1 can have both pro- and anti-apoptotic functions. Cytoplasmic FTH1 can protect hepatocytes and endothelial cells against some apoptotic inducers (Cairo *et al.*, 1995, Theil, 1987). Upregulation of FTH during B-lymphocyte differentiation reduces free iron levels and also increases resistance to oxidative damage (Cozzi *et al.*, 2000, Epsztejn *et al.*, 1999), whilst down-regulation increases free iron and is associated with increased apoptosis (Yang *et al.*, 2002).

Our experiments reveal a slight increase in the incidence of PCD following overexpression of *Fth1*, consistent with a pro-apoptotic function of FTH1 in the early epiblast. An early response to neural inducing signals appears to include upregulation of *Fth1*, which may promote PCD in the vicinity of the neural plate, especially in regions that are free from *Dad1*, which is also induced by signals from the node.

Cell death during neural induction

PCD was originally envisaged as a process of elimination of embryonic cells for "phylogenetic, histogenetic and morphogenetic" purposes (Glucksmann, 1951). There have been numerous studies on the role of apoptosis during many aspects of development, including patterning the early neural crest (Graham *et al.*, 1994, Graham *et al.*, 1993, Homma *et al.*, 1994, Jeffs *et al.*, 1992, Jeffs and Osmond, 1992, Lawson *et al.*, 1999, Wakamatsu *et al.*, 1998). The patterns of PCD reported here confirm an earlier report in the chick, including variability in staining patterns, increased apoptosis at the tip of the streak and left-right asymmetric patterns in a subset of embryos (Sanders *et al.*, 1997b). However we did not observe the increased PCD in the posterior marginal zone and Koller's sickle in pre-primitive streak stage embryos described by others (Hirata and Hall, 2000), and only 6 out of more than 60 embryos analyzed at stages 8-14 showed increased PCD in the notochord as previously reported (Hirata and Hall, 2000).

Previous studies in *Xenopus* (Hensey and Gautier, 1998, Yeo and Gautier, 2003) obtained results reminiscent of those described here, except that they observed high cell death in the neuroectoderm. Our study and another (Sanders *et al.*, 1997b) suggest instead that the chick neural plate is an area of decreased or even absent PCD. At later stages of *Xenopus* development, increased PCD is seen in stripes of primary neurons, sensory placodes and spinal cord (Hensey and Gautier, 1998). There is variability even at stages when the patterns of PCD in the chick become consistent: for example only 67% of *Xenopus* embryos at neural plate stages (stage 13) and 52% of embryos at stage 37 contained more than 5 TUNEL cells per embryo (Hensey and Gautier, 1998). Hensey and Gautier proposed that PCD may contribute to sharpen the boundaries between neural and non-neural territories. Our finding that cells undergoing apoptosis become progressively concentrated at the neural/non-neural border are consistent with this hypothesis.

Several secreted factors have been implicated in the regulation of cell death, especially members of the BMP family. BMP4 is an effector for the localization of apoptosis to rhombomeres 3 and 5 (Graham *et al.*, 1994). Indeed, the lateral neural plate and its descendants, dorsal neural tube and neural crest territory, express a number of BMP family members strongly (Liem *et al.*, 1997, Liem *et al.*, 1995).

In zebrafish, the only data on early PCD concern earlier (Negron and Lockshin, 2004, Yabu *et al.*, 2001) or later (Cole and Ross, 2001) stages of development; for the stages at which it has been studied, the results correspond closely to ours in the chick.

Interestingly lower vertebrates and non-vertebrate chordates do not undergo apoptosis prior to the mid-blastula transition (zebrafish: Ikegami *et al.*, 1999, Negron and Lockshin, 2004; *Xenopus*: Hensey and Gautier, 1997; sea urchin: Voronina and Wessel, 2001), suggesting that transcription is required to control embryonic cell death.

During zebrafish gastrulation, cells undergoing apoptosis have only very low levels of active caspase3, despite *Cas3* being present in the pool of maternal factors as well as being expressed during gastrulation and later in development (Negron and Lockshin, 2004, Yabu *et al.*, 2001). The patterns of *Cas3* expression in zebrafish are comparable to those we report in early chick development (Yabu *et al.*, 2001). Thus, these patterns appear to have been conserved during vertebrate evolution.

We find that the expression patterns of *Cas9* and *Cas3* overlap significantly; *Cas9* is expressed more ubiquitously but at low levels. However there is a region of increased *Cas9* expression that surrounds the prospective neural plate and which seems to correspond to the main sites of cells undergoing PCD. *Dad1* appears to be located within this domain, raising the possibility that Dad1 may contribute to protect cells against apoptosis within the newly induced neural plate, while a narrow region expressing *Cas3* and *Cas9* but devoid of *Dad1* ensures that PCD becomes restricted to the edge of the neural plate (prospective neural crest and placode territory). However we were unable to test this directly using loss-of-function experiments.

Conclusions

Together, our results reveal that neural induction by the organizer (Hensen's node) and normal neural plate development are accompanied by a decrease in the incidence of PCD in the neural plate and an increase in apoptosis at the neural plate border and proximal anterior non-neural ectoderm. This correlates with the induction of *Dad1* expression in the prospective neural plate and the distribution of *Fth1* and Caspases in adjacent regions. Pro- and anti-apoptotic genes are induced as early responses to signals from Hensen's node. While Dad1 may have weak anti-apoptotic properties and Fth1 seems to promote PCD, none of these genes appears to influence cell identity at these stages of development or to be required for early neural plate development.

Materials and Methods

Eggs and embryos

Fertilized Brown Bovan Gold hens' eggs were obtained from Henry Stewart & Co. Fertilized quails' eggs were obtained from B.C. Potter - Rosedean farm. Eggs were incubated at 38ºC and staged according to Hamburger and Hamilton (1951). A modified version of the New technique was used to culture embryos (Stern and Ireland, 1981). Transplantation of Hensen's node was performed at stage 3+/4 as described previously (Stern, 1999, Storey *et al.*, 1992). The graft was placed in the inner 1/3 of the area opaca at or above the level of the host node (Streit *et al.,* 1997). Following transplantation, cultured embryos were incubated at 38ºC in a humid chamber to the required stage.

In situ *hybridization*

Whole-mount *in situ* hybridization with digoxigenin- (DIG) or fluorescein-labelled riboprobes was used, as previously described (Stern, 1998, Streit and Stern, 2001). For *in situ* hybridization with two probes, one probe was detected using Nitro Blue Tetrazolium (NBT) and Bromo-Chloro-Indole-Phosphate (BCIP) as alkaline-phosphatase substrates to yield a deep purple colour. The other probe was detected using BCIP alone to give a light blue colour. In some cases Iodophenyl-Nitrophenyl-Phenyl-Tetrazolium Chloride (INT)-BCIP was used as an alternative to the latter to give a brick-red colour. The chromogens (Roche) were diluted in 100mM Tris-Buffered saline (pH 9.5) containing 50mM Mg^{++} and 1% Tween-20 (NTMT): 4.5µl NBT stock (75µg/ml in 70% dimethylformamide, DMF), 3.5µl BCIP stock (50mg/ml in 100% DMF) per 1.5 ml for dark purple, 7.5µl BCIP stock: (50mg/ml in 100% DMF) per ml for light blue or 7.5µl INT-BCIP per ml for brick-red colour.

Dad1 and *fth1* were cloned into pBlueScript. To generate antisense riboprobes, the plasmids were digested with XhoI and transcribed with T3 Polymerase in both cases. *Cas3* and *Cas9* cDNA (both in pGEMTeasy) were linearised with NcoI and transcribed with SP6.

To distinguish between the almost identical sequences of UbI and UbII, antisense riboprobes were designed to incorporate 3' UTR sequence, which differs between for these genes. The fragments were isolated by PCR from a mixture of two cDNA libraries (stage 2-4 and stages 18-20). The following primers were used:

UbI:

Forward: ACTACAACATCCAGAAG;
Reverse: ATGTGCAACAGAAAAACT.

UbII:

Forward: CCTGTCTGACTACAACATC;
Reverse: GGATGCAAGAACTTTATTG.

PCR fragments were extracted, cloned into pGEM T-easy and transformed into competent bacteria (Promega). After sequencing to validate the clones and to determine the orientation of the insert, they were digested with NcoI and transcribed with SP6.

Whole mount immunohistochemistry

Whole-mount immunohistochemistry was carried out as previously described (Stern, 1998, Streit and Stern, 2001). GFP was detected with rabbit anti-GFP (Molecular Probes) (1:2,500 in blocking buffer), followed by goat anti-rabbit-HRP (Santa Cruz) (1:2,500). Quail cells were detected with monoclonal antibody QCPN (quail cell perinuclear antigen) (developed by Dr B.M. Carlson and obtained from the Developmental Studies Hybridoma Bank, maintained by the Department of Pharmacology and Molecular Sciences, The John Hopkins University School of Medicine, Baltimore, MD 21205, under contract N01-HD-2-3144 from NICHD) as previously described (Storey *et al.*, 1992) using anti-mouse IgG-HRP (Jackson; 1:2,500). FTH1 protein was detected using affinity-purified, rabbit anti-human FTH1 antibody (FERH13-A, Alpha Diagnostic; 1:250). This was raised against a 15aa peptide near the amino-terminus of FTH1, which is 100% conserved in mouse, rat, human, bovine monkey and chicken FTH1. This was detected using goat anti-rabbit-HRP (Santa Cruz; 1:2,500). The HRP-activity was revealed using 3,3'-diaminobenzidine (DAB) and H_2O_2 as previously described (Stern, 1998).

Terminal deoxynucleotidyl transferase mediated nick-end labelling (TUNEL)

The TUNEL method was modified from published protocols (Gavrieli *et al.*, 1992, Wijsman *et al.*, 1993). Embryos were fixed as for *in situ* hybridization, stored in methanol at -20°C and rehydrated gradually to PBT. Embryos were placed in TdT buffer (30 mM Tris pH 7.4,100 mM Na cacodylate, 1 mM $CoCl_2$) for 30 min with gentle rocking. The buffer was replaced with 400 µl of TdT reaction mix (TdT buffer, 0.5 µl DIG-dUTP, 2 µl terminal transferase; Roche) for 4 h at room temperature. After washing in TBST, alkaline-phosphatase conjugated anti-DIG antibody (1:5,000) staining was performed as for *in situ* hybridization.

Wax embedding and sectioning

Stained embryos were rinsed in PBS and dehydrated with alcohol washes (5 minutes in 100% methanol and 10 min in 100% propan-2-ol).

They were then cleared in tetrahydronaphthalene for 30 min, embedded in Fibrowax, sectioned at 8μm and mounted on gelatin-albumen-coated glass slides. After drying, they were de-waxed using Histoclear and mounted in Canada Balsam.

Constructs for electroporation

Misexpression experiments were performed by electroporation using the pCAβ vector (≤-actin promoter, IRES, GFP, with the gene of interest inserted upstream of the IRES). To generate *Dad1* constructs, the vector was digested with BsmBI and ClaI and gel-purified using a Gel Extraction Kit (Qiagen). The reading frame of chick *Dad1* was amplified by PCR from two chick cDNA libraries (stage 2-4 and 18-20, respectively). Primers (Forward: GATCAGCGGCCGCATGTCGGGCACGGCGGG; Reverse: TGCTCATCGATTCAGCCAACAAAATTGATA) incorporating NotI and ClaI sites (underlined above). Ligation was then performed using T4 ligase (Promega) and verified by sequencing. A mutated version of *Dad1* lacking the C-terminal 6 aminoacids (VINFVG) was constructed using the same forward primer and reverse primer TGCTCATCGATTCAGACGAGATGCAGGATGGT, containing a ClaI site).

UbII constructs were generated as for *Dad1*, using primers: GATCAGCGGCCGCGACCAACATGCAGATCTTC (forward) and TGCTAATCGATTCTTCAGTTACCACCCCTG (reverse). For *Fth1*, the primers used were: GATCAGCGGCCGCGATTGGGACGGAACCGGC (forward) and TGCTCATCGATGCCTTCAGCTGTCACTTTCCCCG (reverse). An additional construct, lacking the Iron Regulatory Element (IRE) present in the 5' UTR of *fth1*, the ORF of *fth1* lacking 5' UTR sequences was amplified using primers:
GATCAGCGGCCGCGCCATGGCTACGCCTCC (forward) and TGCTCATCGATGCCTTCAGCTGTCACTTTCCCCG (reverse).

Electroporation

In vivo electroporation (Muramatsu *et al*., 1997), modified for early embryos (Voiculescu *et al*., 2007, Voiculescu *et al*., 2008) was used to introduce the expression constructs described above or fluorescein-labelled morpholinos into selected cells in living whole embryos at stage 3+/4. Three or four 50 msec pulses (500 msec apart) of 5.5-6 Volts were given with a TSS20 pulse generator (Intracel). Following electroporation, embryos were placed onto a vitelline membrane and grown to the desired stage at 38°C. The electroporated embryos were grown for between 6-24 hours and then processed for *in situ* hybridization with various markers including *Sox3*, *Sox2* (Rex *et al*., 1997, Uwanogho *et al*., 1995), *ERNI* (Streit *et al*., 2000), *Dlx5* (McLarren *et al*., 2003, Streit and Stern, 1999, Yang *et al*., 1998), *Pax7* (McLarren *et al*., 2003) and *Gata2* (Sheng and Stern, 1999). After *in situ* hybridization, antibody staining against GFP was used to reveal the electroporated cells.

Design of morpholinos

The chick *Dad1* sequence (GenBank U83627) was confirmed by sequencing a fragment of genomic DNA containing the *Dad1* gene. A fluorescein-tagged morpholino (CACCCGAACCCGCCGTGCCCGACAT) targeting the first 25 bases of the coding sequence was designed by Gene Tools. A fluorescein-labelled control morpholino (sequence CCTCTTACCTCAgTTACAATTTATA) (Gene Tools) was used as a negative control. Each morpholino was used at 2mM in water containing 0.01% Fast Green and 6% sucrose, mixed with empty pCAβ at a final dilution of 1μg/μl (used as a carrier and as an additional marker for the electroporated cells) was introduced into cells by electroporation as described (Sheng *et al*., 2003, Voiculescu *et al*., 2007, Voiculescu *et al*., 2008).

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