FatJ acts via the Hippo mediator Yap1 to restrict the size of neural progenitor cell pools

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SUMMARY
The size, composition and function of the spinal cord is likely to depend on appropriate numbers of progenitor and differentiated cells of a particular class, but little is known about how cell numbers are controlled in specific cell cohorts along the dorsoventral axis of the neural tube. Here, we show that FatJ cadherin, identified in a large-scale RNA interference (RNAi) screen of cadherin genes expressed in the neural tube, is localised to progenitors in intermediate regions of the neural tube. Loss of function of FatJ promotes an increase in dp4-vp1 progenitors and a concomitant increase in differentiated Lim1+/Lim2+ neurons. Our studies reveal that FatJ mediates its action via the Hippo pathway mediator Yap1: loss of downstream Hippo components can rescue the defect caused by loss of FatJ. Together, our data demonstrate that RNAi screens are feasible in the chick embryonic neural tube, and show that FatJ acts through the Hippo pathway to regulate cell numbers in specific subsets of neural progenitor pools and their differentiated progeny.

KEY WORDS: Cadherin, Neural tube, Chick, RNAi, Screen, FatJ, Progenitor cells, Interneurons, Phenotype, Hippo pathway, Notch signalling, Morphogen

INTRODUCTION
Neural progenitor cells are patterned along the dorsoventral axis of the neural tube, proliferate, then migrate laterally and differentiate into defined neuronal classes (Kintner, 2002; Bylund et al., 2003). The tight regulation of patterning, proliferation and differentiation ensures an appropriate balance of progenitor versus differentiated cells that is key to tissue homeostasis. Signalling pathways mediating these events are therefore crucial to the proper size, composition and functioning of the nervous system (Dahmane et al., 2001). However, an outstanding question is that of how appropriate progenitor and differentiated cell numbers are exacted. Recent evidence has implicated the Hippo pathway in such regulation, showing that components of the Hippo signalling pathway govern neural progenitor cell number (Cao et al., 2008).

Cadherin proteins are extracellular proteins that play important roles in cell adhesion and cell signalling (Halbleib and Nelson, 2006). The Fat cadherins comprise a subfamily containing the largest proteins in the superfamiliy (Tanoue and Takeichi, 2005). Drosophila Fat (dFat) was first identified as a putative tumour suppressor gene involved in planar cell polarity and tissue size regulation via the Hippo signalling pathway (Mahoney et al., 1991; Matakatsu and Blair, 2004; Willecke et al., 2006). Of the four vertebrate orthologues of dFat, Fat4 (also called FatJ) shows the greatest homology to dFat (Matakatsu and Blair, 2006) and is expressed in a variety of tissues with active PCP signalling (Rock et al., 2005), including the kidney, where loss of Fat4 alters orientated cell divisions and leads to kidney dysfunction. Fat4 additionally plays a role within the CNS: loss of Fat4 expression in the cerebellum reduces the apical membrane compartment, suggesting a role in apical-basal polarity (Ishiuchi et al., 2009). Furthermore, mouse Fat4−/− embryos display a wider spinal cord than wild-type littermates, suggesting that FatJ/Fat4 might play a role in spinal cord development (Saburi et al., 2008).

We have previously developed a system for robust knockdown of chicken genes (Das et al., 2006). In the present study, we employ this system to knockdown the expression of all cadherin domain-containing genes expressed within the chicken neural tube and thereby analyse cadherin gene function during neural development. Our unbiased approach defines a number of cadherin genes that may play a role in cell patterning, proliferation and differentiation within the developing spinal cord. Of these, we have studied one, FatJ, in detail. FatJ is localised to progenitor cells within intermediate regions along the dorsoventral axis of the neural tube. Our studies reveal that FatJ acts via the Hippo pathway mediator Yap1 to regulate the size of the dp4-vp1 progenitor cell pools and hence differentiated Lim1+/Lim2− interneuron numbers. This study is the first large-scale RNAi screen to be performed in a whole vertebrate organism and reveals an important role for FatJ cadherin, acting via the Hippo signalling mediator Yap1, to control the regionally restricted proliferation and differentiation of specific interneuron classes.

MATERIALS AND METHODS
Microarray analysis of genes expressed in the chicken neural tube
Total RNA extracted from stage 22 chicken spinal cords (100 embryos) was used to interrogate the Affymetrix whole chicken genome chip (ARK Genomics). Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under Accession Number E-TABM-701.
Labelled cDNA targets were generated by reverse transcription of total RNA with modified dNTPs conjugated to aminolyl. A secondary coupling reaction was then carried out to conjugate Cy3 dye to the cDNA. Dye-coupled cDNA was then used to probe genes on the microarray. This led to the identification of ~14,000 expressed genes, which were sorted according to identifiable protein domains using the ENSEMBL chicken genome assembly (version 2.1, May 2006 release). Forty of these genes contained cadherin domains.

**RNAi vectors**

RNAi targeting vectors were designed as previously described (Das et al., 2006). However, the RNAi vectors used in this study were created using an updated RNAi vector (pRFPRNAiC) containing a MluI site instead of the ApaI site in the microRNA expression cassette. Initially, two siRNA targets were generated for each cadherin and subsequently a third siRNA target was generated for each cadherin to ensure a maximal probability of the vectors producing a gene-specific knockdown. It has been suggested that employing at least two independent RNAi vectors for each target gene should significantly reduce the incidence of potential off-target effects (Echeverri et al., 2006). The target siRNA sequences for each cadherin and for Yap1 and Tead4 are listed in Table S1 in the supplementary material. All the RNAi plasmids described here are available as a community resource from our clone distributors (ARK-Genomics.org).

**RNAi screening**

Two RNAi vectors for each gene were pooled and electroporated into stage 11-12 neural tubes. A third RNAi vector for each gene was subsequently electroporated into separate embryos. Electroporation procedure was as previously described (Das et al., 2006); however, the maximum DNA concentration in all electroporations was 0.5 μg/μl. After 48 hours of incubation, the embryos were analysed for defects caused by RNAi targeting of the particular cadherin. Primary phenotypic analysis was performed by analysing the location and morphology of RFP-positive cells.

**Embryo fixation, immunohistochemistry and in situ hybridisation**

Embryo fixation, sectioning and immunohistochemistry were performed as previously described (Das et al., 2006). Antibodies used were against Pax6, Pax7, Pax2, Evx1, Nkx2.2, Isl1, Lim1/Lim2, Lmx1b, N-cadherin, Nkx6.1 and En1 (DSHB); mouse anti-β-catenin (Sigma); rabbit anti-Dbx1 (Pierani et al., 1999); anti-Chx10 (S. Morton, Columbia University, NY, USA); rabbit anti-Pax6 (Covance); rabbit anti-Olig2 polyclonal and rabbit anti-RFP polyclonal (Chemicon); rabbit anti-phospho-histone H3 polyclonal (Upstate); rabbit anti-GFP polyclonal (BD Bioscience); anti-Crb2 (P. Rashbass, University of Sheffield, UK); rabbit anti-pYap1 polyclonal (Cell Signaling Technology); and Alexa405-, Alexa488- and Alexa555-conjugated secondary antibodies were obtained from Molecular Probes (Invitrogen). Whole-mount in situ hybridisation was performed as previously described (Ohayama et al., 2005; Das et al., 2006) using digoxigenin (DIG)-labelled (Roche) RNA probes. DIG-labelled antisense riboprobes were generated from chicken EST plasmids ChEST187n19 (Fat1); ChEST427d5 (Yap1) and ChEST580f24 (Tead4) [by linearization with NotI and transcription with T3 RNA polymerase (Promega)]. After sufficient staining development, embryos were cryosectioned at 30 μm as described previously (Das et al., 2006).

**BrdU incorporation studies**

Neural tubes were electroporated as described above. After 48 hours incubation at 38°C, 50 μl of 0.01 M BrdU (Sigma) was injected under the vitelline membrane, the egg resealed and placed at 38°C. After 30 minutes incubation at 38°C, 50 μl of 0.01 M BrdU (Sigma) was injected under the vitelline membrane, the egg resealed and placed at 38°C. After 30 minutes

![Fig. 1. A range of phenotypes are observed following loss of cadherin function. Expression of red fluorescent protein (RFP) from RNAi vectors allows cell migration and morphology to be assessed. (A) RNAi-mediated knockdown of Fat2 cadherin causes an accumulation of electroporated cells in the mantle zone (mz) of the neural tube. (B) A similar phenotype is observed after knockdown of Notch1 (Das et al., 2006), whereas cells targeted with luciferase RNAi (C) are located throughout the neural tube in both ventricular zone (vz) and mantle zone. (D) Fat1 RNAi results in a reduction in size and cell number (32% fewer cells) in the electroporated side of the neural tube. (E) Expression domain of Pax6 is reduced relative to the contralateral side. (F) Loss of N-cadherin produces a decrease in cell number on the electroporated side, together with disruption of the apical surface of the neural tube, as marked by loss of β-catenin expression. (H) Protocadherin 19 RNAi leads to a 50% increase in the number of cells on the electroporated side of the neural tube and an expansion of Pax6 expression compared with the contralateral side (I). Some electroporated cells also migrate into the unelectroporated side of the neural tube (arrows in F), suggesting there may be some loss of roof plate integrity. (J) RNAi-mediated knockdown of protocadherin 10 leads to a dorsal shift in expression of Pax6 (J) and Olig2 (K). (L) Knockdown of Fat1 RNAi-mediated knockdown of protocadherin 10 leads to a dorsal shift in expression of Pax6 (J) and Olig2 (K).
further incubation, embryos were harvested, fixed and cryosectioned as previously described (Das et al., 2006) and immunohistochemistry was performed using 1/1000 mouse anti-BrdU monoclonal antibody (Sigma).

Microscopy and image acquisition
Epifluorescence images were obtained using a Leica DM5000B upright microscope and confocal images were obtained using a Zeiss LSM510 Meta. Section in situ images were obtained under Nomarski optics on a Leica DM-R microscope using a Leica digital camera.

Co-electroporation of Notch intracellular domain
The pCS2-ICD plasmid expressing the intracellular domain of mNotch1 (NICD) was kindly gift from D. Henrique (IMM, University of Lisbon, Portugal) and has been previously shown to constitutively activate Notch1 signalling (Kopan et al., 1996). The plasmid was co-electroporated with the RNAi vectors [1 µg/µl NICD + 0.5 µg/µl of the relevant RNAi vector(s)] at stage 11-12 and the resulting phenotype was analysed at stage 25 as detailed above.

Western blotting
Neural tubes were electroporated at stage 11-12 with Fat2 RNAi or Luc RNAi vectors and harvested after 72 hours incubation at 38°C. The intermediate region of the electroporated side of six or more neural tubes was dissected and lysed in Reporter Gene Lysis buffer (Roche). Each protein lysate (20 µg) was separated by SDS-PAGE in a 10% polyacrylamide gel, transferred to a nitrocellulose membrane and western blotting was performed using 1/1000 rabbit anti-pYap1 antibody (Cell Signalling Technology) in 10% BSA/1×TTBS followed by incubation with 1/10,000 anti-rabbit HRP-conjugated secondary antibody and detection by ECL. Each membrane was subsequently incubated with 1/5000 mouse anti-tubulin antibody (Sigma) and 1/5000 rabbit anti-RFP polyclonal (Chemicon) to evaluate protein amounts loaded and degree of electroporation with RNAi vectors. Signal density was determined using Quantity One software (Biorad).

RESULTS
Cadherin genes influence neural tube development
We identified cadherin domain-containing genes (cadherins) expressed in the embryonic chick neural tube by probing whole chicken genome microarrays with RNA isolated from stage (St) 22 neural tubes. The analysis identified 40 genes containing at least one cadherin domain expressed in the neural tube (see Table S1 in the supplementary material). To investigate the role of each cadherin in neural tube development, we generated three RNAi vectors for each gene (see Table S2 in the supplementary material), electroporated each into St 11-12 neural tubes and analysed embryos after 48 hours (St 25). As a primary phenotypic analysis, we examined the location and morphology of electroporated cells, visualised through red fluorescent protein (RFP) expression. As a secondary screen, expression patterns of the progenitor markers Pax6, Pax7 and Nkx2.2, and differentiation markers Islet1, Lim1/Lim2 and Lmx1b were analysed. Electroporated cells were compared with cells on the contralateral unelectroporated side and with cells electroporated with an RNAi control vector targeting luciferase. Electroporation of RNAi vectors targeting 18 cadherins caused no detectable phenotype (see Table S1, Fig. S1 in the supplementary material). However, 22 others gave phenotypes that could be broadly classified (see Table S1 in the supplementary material).

Knockdown of four cadherins (Fat2, brain cadherin, VE-cadherin and cadherin 18) resulted in accumulation of RFP+ cells in the mantle zone, a markedly different effect to that normally observed (Fig. 1A-C; see Fig. S1 in the supplementary material). This effect phenocopies knockdown of Notch1, where premature differentiation of cells results in their migration from the ventricular to the mantle zone (Fig. 1B, Fig. 2B) (Das et al., 2006), raising the possibility that these cadherins may function in the same pathway. We investigated this possibility by co-electroporating a constitutively active form of Notch1 (Notch intracellular domain, NICD), shown previously to rescue the Notch RNAi phenotype (Kopan et al., 1996), with the Fat2 RNAi construct. Co-electroporation of NICD rescues the premature differentiation caused by Fat2 RNAi, and electroporated cells were now found throughout the neural tube in both the ventricular and mantle zones (Fig. 2D). This suggests that Fat2 functions in the same pathway as Notch1 to control neural progenitor differentiation.

Knockdown of 14 cadherins caused a reduction in size of the electroporated side of the neural tube (Fig. 1D,E; see Fig. S1 in the supplementary material), suggesting a decrease in cell number. No changes in dorsoventral (DV) patterning were detected; instead, the domain of each homeodomain protein examined was small relative to the contralateral side (Fig. 1G; see Fig. S1 in the supplementary material); this phenotype was not observed following introduction of luciferase RNAi (see Fig. S1 in the supplementary material). Knockdown of N-cadherin caused a similar defect, and additionally caused a loss of β-catenin expression at the apical surface of the neural tube (Fig. 1E,H) as observed previously for a mutation in this gene in zebrafish (Lele et al., 2002). By contrast, knockdown of protocadherin 19 and cadherin 10 resulted in an increased number of cells in the neural tube and enlargement of the electroporated side. Concomitantly, there was a relative expansion of the domain of expression of each homeodomain protein examined (Fig. 1F,I; see Fig. S1 in the supplementary material).

A minor set of cadherins, when downregulated, caused very localised effects along the DV axis. RNAi-mediated knockdown of protocadherin 10 (Pcdh10) led to a change in DV patterning with...
a dorsal shift in the expression of both Pax6 (Fig. 1J) and Olig2 (Fig. 1K). Neither marker appeared to be expanded; instead, the entire domain of each appeared dorsally shifted. The size of the electroporated side of the neural tube was unchanged compared with the contralateral side, suggesting that the effects observed were likely to be due to a change in patterning.

Finally, FatJ RNAi appeared to produce a small, but robustly reproducible, increase in the number of Lim1+/Lim2+ cells. This phenotype was apparent in only a subset of Lim1+/Lim2+ cells, notably those in medial regions along the dorsoventral axis (Fig. 1L, M, arrows). Lim1+/Lim2+ cells in dorsal regions of the neural tube appeared unaffected by electroporation of FatJ RNAi vectors, whereas those in intermediate regions were expanded in number (Fig. 1N). Together, this raises the possibility that FatJ knockdown affects cell proliferation/differentiation, but that its effects are localised to a regionally defined domain of the neural tube. We therefore focused our attention on this cadherin, to confirm the increase in Lim1+/Lim2+ cells and clarify the mechanism that governs Lim1+/Lim2+ cell numbers.

We first confirmed that all three FatJ RNAi vectors, which target different regions of FatJ mRNA (Fig. 3; see Table S2 in the supplementary material), knock down FatJ expression and confirmed that luciferase RNAi vector has no effect. To further ensure that the effects of the FatJ RNAi vectors were not mediated by an ‘off-target’ effect (see Echeverri et al., 2006), we compared the ability of each FatJ RNAi vector to increase production of Lim1+/Lim2+ interneurons (Fig. 3G-I). Each vector caused at least a 20% increase in the number of cells expressing Lim1+/Lim2+, with the most effective vector (FatJ RNAi C) producing an average of 32% more Lim1+/Lim2+ cells (Fig. 3M). We conclude that FatJ regulates the number of Lim1+/Lim2+ neurons in the neural tube.

Given that Lim1/Lim2 is expressed in a wide set of interneurons (dl2, dl4, dl6, v0 and v1 subclasses), we next defined more precisely which interneuron types were affected by FatJ RNAi, by analysing embryos for Isl1, Pax2, Lmx1b, Evx1, En1 and Chx10 expression, which mark dl3, dl4+6, dl5, v0, v1 and v2 interneurons, respectively (Fig. 4). This analysis revealed that interneurons in domains dl4, dl5, dl6, v0 and v1 were affected (Fig. 4B-F), whereas those in domains dl3 and v2 were not (Fig. 4A, G). We conclude that FatJ knockdown results in the specific expansion of differentiated dl4-v1 interneurons.

**FatJ expression is spatially restricted**

One possible explanation for the effect of FatJ RNAi in only a subset of Lim1+/Lim2+ cells is that FatJ itself is spatially restricted to intermediate regions of the neural tube. To examine this, we analysed FatJ mRNA expression over the period St10-
Fig. 4. Detailed analysis of increase in interneurons following FatJ knockdown. (A) Following loss of FatJ, there is no change in the number of cells expressing the marker Islet1 (Isl1), which marks the dI3 class of interneurons. (B-F) Loss of FatJ results in an increase in specific interneuron number, relative to the contralateral side: 9.52% more Pax2+ cells (B; dI4 and dI6 interneuron); 16.11% more Lmx1b+ cells (C; dI5 interneurons); 15.24% more Lim1+/Lim2+ cells (D; dI2, dI4, dI6, v0 and v1 interneuron classes; 20.00% more Evx1+ cells (E; marking v0 interneurons); 17.91% more En1+ cells (F; v1 interneurons). (G) Loss of FatJ does not increase the number of cells expressing Chx10 (v2 interneuron class). Therefore, the increase in interneuron number is observed from the dI4 to v1 interneurons. Average cell counts for each marker on both sides of the neural tube are shown graphically on the right of the figure (error bars show s.e.m.). Statistical significance of the cell counts was determined using a paired Student's t-test.
St22 and found that its expression is restricted to intermediate regions along the DV axis. Expression is limited to ventricular/subventricular zone areas and appears to correlate with dp4-vp1 progenitor regions, overlapping with expression domains for progenitor markers Pax6, Pax7 and Dbx1, but not Nkx6.1 (Fig. 5A-F, Fig. 3J-L). This pattern of expression is reminiscent of that of Pax6, which is regulated by Shh and Wnt/BMP signalling and the Gli activity gradient (Briscoe et al., 2000; Timmer et al., 2002).

**FatJ knockdown alters progenitor cell number**

Two alternative possibilities could account for the enhanced differentiation of dI4-v1 interneurons after FatJ knockdown. First, a decrease in FatJ activity could lead to acceleration in the differentiation of progenitor cells in the dp4-vp1 domain. In this case, knockdown of FatJ should lead to a decrease in proliferating progenitors within these domains. An alternate possibility is that FatJ governs the proliferating progenitor cells themselves, a decrease in its activity leading to greater numbers of progenitor...
cells, and hence greater numbers of differentiated interneurons. In this case, knockdown of FatJ should lead to an increase in proliferating progenitors.

To distinguish these possibilities, and determine whether FatJ expression is required for differentiation, or for the establishment/maintenance of proliferating progenitors, we quantified the number of progenitor cells in defined subdomains along the DV axis of the neural tube after FatJ RNAi. Loss of FatJ did not affect the general pattern of any of the markers examined (Fig. 5G,H; Fig. 6A,B). Moreover, loss of FatJ expression did not affect the number of progenitor cells in the vp2 domain (judged by Nkx6.1 expression; Fig. 5G,M; Table 1), the vp3 domain (Nkx2.2 expression; Fig. 6B; Table 1) or the dp3 domain (low expression of Pax6; Pax7 expression) (Fig. 5H, Fig. 6A, Table 1). Thus, progenitor cells lying outside the FatJ expression domain were unaffected by its reduction. By contrast, a small but consistent increase (10-20%, Table 1) in the number of Dbx1+ (Fig. 5G,N), Pax6 (strong positive) (Fig. 5H,O) and Irx3+ cells (Table 1) was detected. This suggests that reduction in FatJ expression leads to an increased number of progenitors in the dp4-vp1 domains of the neural tube.

To begin to address the mechanism underlying the increase in progenitor number, we analysed BrdU incorporation. A 14.65% increase in the number of proliferating cells was detected in the ventricular zone after FatJ RNAi (Fig. 5I,K; Table 2). Similarly, we observed a 21.92% increase in the number of mitotic cells marked by phospho-histone H3 (Fig. 5J,L; Table 2). These data suggest that the increased number of differentiated interneurons can be explained by an increased number of progenitors within the corresponding progenitor pool. To further test this idea, we performed double labelling analyses with Pax6 and En1. An increased number of En1+ cells was detected after FatJ knockdown, but no double-positive cells were detected (Fig. 5P), indicating that En1+ cells differentiated properly and did not simply express En1 precociously.

FatJ is the closest orthologue of the Drosophila Fat (dFat) gene, which plays a crucial role in planar cell polarity (Fanto et al., 2003; Matakatsu and Blair, 2006) and interacts with Pals1 to organise the apical membrane domain (Ishiuchi et al., 2009). To determine whether the increase in progenitor cell number is caused by defects in progenitor cell polarity, we examined expression of the apical proteins N-cadherin, Crumbs2 and Par3. Loss of FatJ did not appear to affect expression of any of these markers (Fig. 6C,D; data not shown), suggesting that apico-basal polarity is unaffected by loss of FatJ. Our data indicate that FatJ limits the number of progenitor cells within its domain of expression through a mechanism other than a disruption in apicobasal polarity.

Table 1. Number of cells expressing progenitor markers after FatJ RNAi

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<th>Marker</th>
<th>Number of sections</th>
<th>Electroporated</th>
<th>Unelectroporated</th>
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<th>s.e.m. (unelectroporated)</th>
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FatJ controls cell proliferation through the Hippo mediator Yap1

*Drosophila* Fat controls tissue size by activating the Hippo pathway, leading to phosphorylation of the transcriptional activator Yorkie (Yki) (Willecke et al., 2006). Yki normally associates with the transcription factor Scalloped (Sd) to promote cell proliferation (Wu et al., 2008). Activation of the Hippo pathway sequesters Yki in the cytoplasm via Yki phosphorylation or by direct binding of Yki by the Hippo pathway components Expanded and Warts, thereby preventing association of Yki with Sd and stopping cell proliferation (Zhang et al., 2008; Oh et al., 2009). Intriguingly, the Hippo pathway has recently been shown to control neural progenitor cell number in the neural tube and expression of dominant repressor versions of the vertebrate orthologues of Yki and Sd (Yap1 and Tead1, respectively) can produce an increase in the number of Lim1+/Lim2+ cells, similar to the phenotype observed in this study (Cao et al., 2008). This raises the possibility that FatJ acts through the Hippo pathway to control the number of cells within the dp4-vp1 domain. To test this, downstream components of the Hippo pathway were knocked down simultaneously with FatJ knockdown. RNAi mediated knockdown of Yap1 was confirmed by in situ hybridisation: electroporation of Yap1 RNAi constructs produced a significant loss of Yap1 expression within cells expressing the RNAi constructs (Fig. 7A), whereas expression of luciferase RNAi vectors had no effect on Yap1 expression (Fig. 7B). Notably, Yap1 expression is observed solely in the ventricular/subventricular zones of the neural tube; therefore, the FatJ expression domain comprises a subset of Yap1-expressing cells. Knockdown of Yap1 (Fig. 7C,F) and Tead4 (the closest vertebrate orthologue of Sd, not shown) alone had no significant effect on the number of cells expressing Lim1/Lim2. However, knockdown of Yap1 or Tead4 together with FatJ (Fig. 7D,F) rescued the increase in Lim1+/Lim2- cells detected after knockdown of FatJ alone (Fig. 7E,F). To further confirm this rescue, dominant negative (DN) Yap1 or Tead4 constructs [detailed in Cao et al. (Cao et al., 2008)] were co-electroporated with the FatJ RNAi vector. In both cases, these were able to rescue the increase in Lim1+/Lim2- cells observed after knockdown of FatJ alone (Fig. 7G).

To further investigate regulation of the Hippo pathway by FatJ, we compared the levels of phosphorylated Yap1 (pYap1) after FatJ or Luciferase RNAi. Western blotting revealed decreased levels of pYap1 following FatJ RNAi when compared with Luc RNAi control samples (Fig. 7H). Similarly, decreased pYap1 levels were observed within the FatJ expression domain on neural tube sections after FatJ RNAi but not after Luciferase RNAi electroporation (Fig. 7I). Notably, the decrease in pYap1 was most pronounced at the lateral edge of the ventricular zone (arrows in Fig. 7I) adjacent to the mantle zone.

These data suggest that loss of FatJ signalling causes a decrease in phosphorylation of the Hippo mediator Yap1. The upstream Hippo pathway kinases Mst1/2 and Lats1/2 have been shown to regulate neuronal progenitor proliferation by inhibiting Yap1 activity through Yap1 phosphorylation (Cao et al., 2008); however, we were unable to detect any reduction in the levels of pMst1/2 after FatJ RNAi. Therefore, we are unable to distinguish whether FatJ operates through the established Hippo pathway, or via a parallel pathway that links FatJ and pYap1. Nonetheless, taken together, our data show that loss of downstream Hippo components can rescue the defect caused by loss of FatJ and implicates FatJ-control of Hippo pathway mediators as a mechanism for limiting the number of interneurons differentiating in the dp4-vp1 region of the neural tube.

**DISCUSSION**

Our analyses provide proof-of-principle for large-scale RNAi screening in the chick neural tube and provide insight into those cadherins whose RNAi-mediated knockdown leads to early defects in size, proliferation, cell differentiation and patterning of gene expression. The large size of cadherin genes can preclude analysis of their roles through gain-of-function approaches, highlighting further the importance of the RNAi approach. Our studies complement and support studies that document a role for cadherin genes in early neural tube development, for example, showing that N-cadherin is required to maintain the integrity of the neuroepithelium (Lele et al., 2002). In addition, our studies highlight the temporal effects of cadherins in neural tube development: our screen did not reveal early roles for cadherins previously shown to affect later development of the spinal cord, including late migration of neural crest cells (Coles et al., 2007), sorting of cell pools (Price et al., 2002) and synapse formation (Bao et al., 2007).

Our detailed analysis of the FatJ knockdown phenotype provides insight into a novel early role for cadherin function within the CNS, revealing a mechanism to govern the balance of regionally restricted progenitor pool cells and cognate interneuron numbers. Local interneuron circuits play a major role in coordinating the sensory-motor circuits that characterise the spinal cord. The number of interneurons of a particular class is likely to be crucial in achieving appropriate function of selective sensory-motor circuits, and our studies provide insight into the manner in which appropriate numbers of Lim1+/Lim2+ interneurons are generated.

Our data show that FatJ restricts the size of progenitor pools in which it is expressed. Loss-of-function of FatJ leads to a consistent increase in progenitor cell number. These appear to differentiate along their normal route, as evidenced by a consistent increase in appropriate differentiated interneuron subtypes. Mouse Fat4-/- embryos display a wider spinal cord than do wild-type littersmates, suggesting that the role of FatJ/Fat4 in neural progenitor expansion might be conserved across vertebrate species (Saburi et al., 2008). Intriguingly, our data reveal that FatJ restricts progenitor cell numbers via mediators of the recently discovered Hippo signalling pathway. Originally identified in *Drosophila*, activation of the Hippo pathway by dFat prevents the transcriptional activation of genes such as CyclinE and Diap1 that promote cell proliferation and prevent apoptosis (Wu et al., 2003), and in this manner, prevents excessive tissue growth. The Hippo pathway is conserved in vertebrates with one or more orthologues of all components in the pathway. Several of these orthologues can rescue the corresponding *Drosophila* mutant phenotype (Wu et al., 2003; Lai et al., 2000).
et al., 2005; Wei et al., 2007), suggesting a conserved role in growth control. Mutation of many of these genes has been implicated in human cancers (McClatchey and Giovannini, 2005; Harvey and Tapon, 2007; Yokoyama et al., 2008), Fat4/FatJ has recently been implicated as a breast tumour suppressor gene (Qi et al., 2009), and Yap1 expression and nuclear localisation is upregulated in Shh-associated medulloblastomas (Fernandez et al., 2009). Recent studies show that the Hippo pathway controls the number of neuronal progenitors in the neural tube by influencing cell proliferation and apoptosis (Cao et al., 2008). Our studies now reveal that regulation of the Hippo pathway mediator Yap1 in the vertebrate neural tube occurs through FatJ, the closest vertebrate homologue to dFat. Together, our data suggest that FatJ mediates a robust mechanism for the acquisition of appropriate numbers of cells in progenitor pools, and hence appropriate numbers of distinct interneuron subtypes, along the DV axis of the neural tube.

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