Neurons derive from the more apical daughter in asymmetrically fated divisions in the zebrafish neural tube

Paula Alexandre¹, Alexander M. Reugels², David Barker¹,
Eric Blanc¹ and Jonathan D. W. Clarke¹

1- MRC Centre for Developmental Neurobiology, King's College London, New Hunt's House, Guy's Hospital Campus, London SE1 1UL, UK.

2- Institut für Entwicklungsbiologie, Universität zu Köln, 50923 Köln, Germany.

Correspondence should be addressed to JDWC (jon.clarke@kcl.ac.uk).

Abstract

In the developing CNS asymmetric cell division is critical to maintain the balanced production of differentiating neurons while also renewing the population of neural progenitors. In invertebrates this process depends on asymmetric inheritance of fate determinants during progenitor divisions. A similar mechanism is widely believed to underlie asymmetrically fated divisions in vertebrates but compelling evidence for this is missing. We use live imaging of individual progenitors in the intact zebrafish embryo CNS to test this hypothesis. We provide the first direct evidence that asymmetric inheritance of a subcellular domain is strongly correlated with asymmetric daughter fates and reveal an unexpected feature of this process. The daughter cell destined to become a neuron is derived from the more apical of the two daughters, while the more basal daughter inherits the basal process and replenishes the apical progenitor pool.

Asymmetrically fated division generates two daughters with distinct fates and is a fundamental mechanism to generate cell diversity during embryogenesis. During early brain development regulation of this process is critical to maintain the balanced production of differentiating neurons while also renewing the population of neural progenitors. Currently the cellular and molecular mechanisms that regulate

asymmetrically fated divisions during vertebrate neurogenesis remain poorly understood. Understanding how this process works may have important implications not only for development of the nervous system but also for other populations of progenitors and stem cells in embryonic and adult tissue. Much of the molecular analyses of vertebrate neurogenesis are based on testing for analogous mechanisms to those described in central and peripheral nervous systems of *Drosophila* embryos. In particular the possibility that asymmetrically fated divisions might be regulated by asymmetric inheritance of fate determinants (such as Numb) has been hotly pursued in vertebrate brains. The existing information on regulation of vertebrate neurogenesis is derived either from *ex vivo* slice or explant preparations^{1,2,3,4,5,6,7,8}, from dissociated cell culture^{9,10,11,12,13}, or by extrapolation from static images of fixed tissues^{12,13,14,15,16,17}. This diversity of approaches and systems has not generated a consensus for the cellular and molecular mechanisms that guide asymmetric fates during neurogenesis. Although some data is consistent with the hypothesis that asymmetric inheritance could determine asymmetric fates^{9,15} other work suggests that even our knowledge of the distribution of putative fate determinants within vertebrate progenitors is insufficient to allow interpretation of the available data¹⁶. Perhaps the most compelling evidence for asymmetric inheritance in vertebrate neural progenitors is the differential distribution of the apical domain in neurogenic and proliferative divisions^{14,17}. These data, chiefly obtained from static images, show that the apical domain of progenitors can be asymmetrically inherited during neurogenic divisions and underlie the hypothesis that this asymmetry drives the asymmetry of daughter fates^{14,18}. However, the critical question of whether asymmetric inheritance correlates directly with particular daughter cell fates *in vivo*, has not been answered up to now.

To answer this question our work uses *in vivo* imaging in an intact vertebrate neural tube. Our aim is to test the hypothesis that inheritance of the apical domain of neural progenitors is related to the asymmetric fate of daughter cells (Supplementary Fig. 1). By using GFP tagged versions of the apical protein Par3 in the transparent zebrafish embryo we show for the first time a direct correlation between asymmetric inheritance of the apical progenitor domain and asymmetric daughter fates. We also observe allocation of daughter fates in asymmetric divisions is not stochastic and surprisingly the daughter that

inherits the apical domain becomes the neuron, while the more basal daughter inherits the basal process and replenishes the apical progenitor pool. Loss of function experiments support this observation by revealing that Par3 and aPKC are required to promote neurogenic divisions. Our live imaging data thus reveal novel aspects of the cellular and molecular mechanisms that underlie asymmetrically fated divisions in a vertebrate neural tube.

Results

To follow progenitor divisions and assess fates of daughter cells *in vivo*, we performed confocal timelapse microscopy in intact zebrafish embryos in the region of the caudal hindbrain and anterior spinal cord. Cells were labelled by injecting mRNAs coding for membrane-GFP and H2B-RFP and imaged from 20 hours post fertilization (hpf) for up to 30 hours. Embryos of the tg(HuC:GFP) line were used to confirm the identity of neurons. A progenitor fate (P) was assigned if a daughter divided again during the timelapse and neuronal fate (N) assigned if the daughter developed HuC:GFP expression in the cytoplasm and nuclei (white arrow in Fig. 1). This method allowed unambiguous identification of divisions generating two progenitors (PP- symmetric proliferative divisions), divisions generating a neuron and a progenitor (NP- asymmetric divisions) (Fig. 1 and Movie 1) and divisions generating two neurons (NN- symmetric neurogenic divisions) (not shown).

Par3-GFP outlines the apical footprint of progenitors

To determine the inheritance of the apical domain of progenitors during division we have used live confocal imaging to monitor the distribution of the fusion protein Par3-GFP¹⁹, in the posterior hindbrain. Par3-GFP is expressed in a ring-like pattern around the apical surface of progenitors (Fig. 2a, 2b, 2c, 2e). To identify the location of the Par3-GFP domain we determined that it colocalizes with ZO-1 and aPKC, and the strongest domain of F-actin (Fig. 2c-e). This suggests the Par3-GFP ring lies at the level of junctional belts that sit at the interface between apical and basolateral domains in neuroepithelial cells. We therefore refer to the region outlined by and including the Par3-GFP ring as the apical footprint. To determine that Par3-GFP expression itself does not alter the size of the

apical footprint, we measured their size using ZO-1 expression in Par3-GFP expressing and non-expressing cells. They have identical size distributions (Supplementary Fig. 2).

Apical footprint can be symmetrically or asymmetrically inherited

To investigate the inheritance of the apical footprint by daughter cells during division, we mosaically expressed the fusion protein Par3-GFP and analysed its distribution in dividing progenitors. We defined symmetric inheritance as both daughter cells receiving some brightly polarized Par3-GFP and asymmetric inheritance if only one daughter receives the Par3-GFP domain. We collected z-stacks at each time point in the time-lapse sequence and 3-D reconstructions from this data demonstrate that the apical footprint can be either symmetrically (29/50 divisions; Fig. 3a and Movie 2) or asymmetrically inherited (21/50 divisions; Fig. 3b and Movie 3, see also Movie 4 for 3D reconstruction). To confirm that inheritance of Par3-GFP is a reliable readout for inheritance of endogenous junctional protein we compared it to inheritance of endogenous ZO-1. We mosaically injected embryos with Par3-GFP and H2B-RFP, fixed them at 22hpf and used an antibody to detect ZO-1 expression in Par3-GFP expressing cells. We find asymmetric inheritance of Par3-GFP matches asymmetric inheritance of endogenous ZO-1 (Fig. 3c and Supplementary Fig. 3 for all z- levels).

We also find the apical band of Par3-RFP and basolateral marker Numb-GFP have adjacent complementary domains of distribution that are maintained through mitosis (for more details see Supplementary Fig. 4, Movies 5 and 6). These results support the view that Par3 fusion proteins are a good read out of an endogenous apical domain that includes ZO-1 and aPKC.

To assess whether asymmetric inheritance of the apical footprint is determined by the orientation of the cleavage plane we analysed Par3-GFP distribution together with cleavage orientation. We find asymmetric inheritance of Par3-GFP can occur both when the cleavage plane is close to perpendicular to the apical surface (Figures 3b and 3d) as well as when the cleavage plane is oblique or close to parallel to the apical surface (Figure 4a). Thus asymmetric inheritance of the apical footprint must be regulated by factors other than cleavage plane alone, an observation that supports those of Kosodo et al (2004).

Inheritance of apical footprint correlates with cell fates

To determine whether daughter cell fate is correlated with asymmetric inheritance of Par3-GFP, dividing cells and their daughter fates were followed by time-lapse in tg (HuC:GFP) embryos (Fig. 4a, Movie 7 for time lapse sequence and Movie 8 for 3D reconstruction of asymmetric inheritance in this division). These movies revealed that if both daughters were progenitors (PP- symmetric proliferative divisions) then Par3-GFP was usually inherited symmetrically (24/32 divisions), whereas in divisions that generated one neuron and one progenitor (NP- asymmetric divisions) the Par3-GFP was usually asymmetrically inherited (12/15 divisions) (Fig. 4b). We observed a very small number (3/50) of symmetric neurogenic divisions (NN) at this period of neurogenesis. These data demonstrate that asymmetric inheritance of the apical footprint is largely predictive for asymmetric daughter fates but not obligatory thus lending partial support to the previously suggested hypothesis^{14,18,20}.

Oblique cleavage planes favour asymmetrically fated divisions

If asymmetric inheritance of the apical footprint plays an important role in determining asymmetric fates of daughter cells, then manipulations that increase asymmetric inheritance of the apical domain, such as increasing the proportion of divisions with parallel cleavage planes^{1,21,22}, should result in more asymmetrically fated divisions. We noticed that Par3-GFP expression itself has a mild destabilizing effect on cleavage orientation. For cells expressing membrane-GFP 85% (40/47 divisions) cleave within 15° of perpendicular to the apical surface (Fig. 5a); whereas for cells expressing Par3-GFP, although the majority (55%, 43/78) still cleave close to perpendicular, a significant increase in oblique or parallel cleavages is seen (35/78 cleave more than 15° away from perpendicular) (Fig. 5b). When we assessed the overall proportion of divisions generating symmetric and asymmetric fates we could not detect any significant difference between Par3-GFP expressing cells and control cells expressing membrane-GFP (Fig. 5c). However in divisions that we were able to both measure cleavage plane and determine daughter fates (n=26), we find cleavages within 15° of perpendicular mainly produce daughters with symmetric proliferative fates (12PP:3NP:1NN) while cleavages more than

15° from perpendicular generate an increased proportion of asymmetrically fated divisions (4PP:5NP:1NN) (Fig. 5b). Since oblique and perpendicular cleavage planes should favour asymmetric inheritance of the apical footprint this data is consistent with the hypothesis that asymmetric inheritance of the apical footprint favours asymmetric fates.

In asymmetric divisions the neuron inherits the apical footprint

To determine whether inheritance of the apical domain correlates with a particular daughter cell fate, or whether the asymmetric fates are randomly assigned to daughters, we followed individual daughters from divisions where the apical footprint is asymmetrically inherited (see Movies 7 and 9). It has been proposed that daughters inheriting the apical domain would become progenitors and the more basal daughters with no apical attachment would become neurons^{1,18,20}. Our analyses in fact reveal the reverse is true. In 13/14 asymmetrically fated divisions the daughter that inherits apical Par3-GFP becomes the neuron and the progenitor derives from the Par3-GFP negative cell (Fig. 4a, Movies 7, 8, 9 and 10; Supplementary Fig. 5 and 6). This observation contradicts the previous hypothesis and implies the neuronal daughter will remain attached to the apical surface for some period following mitosis, while the Par3-GFP-negative daughter must either be initially detached from the apical surface and then rebuild a new apical attachment or must reinforce any apical contacts that remain outside the Par3-GFP domain to replenish the apical progenitor pool. We observed the neuronal daughters can remain attached to the apical surface for up to 1 cell cycle length after mitosis, during which period their nucleus migrates to the mantle layer (white asterisks in Movies 1 and 7). To investigate the behaviour of non-apical daughters we imaged dividing cells labelled either with membrane-GFP (to ensure expression of Par3 fusion proteins does not alter this behaviour) or with Par3-RFP. In cells expressing membrane-GFP that divided with oblique cleavage planes we observed the more basal daughter does appear to form a new apical attachment soon after division (white arrow in Fig. 6a and Movie 11) and becomes a progenitor (blue asterisk and blue arrow in Fig. 6a). We also observed that daughter cells that do not inherit Par3-RFP are able to generate a bright polarized Par3-RFP domain at the apical surface within 10 minutes (white arrows in Fig.

6b and see Movie 12).

In summary these data reveal two novel aspects of asymmetrically fated divisions. First, contrary to previous predictions, the daughter that inherits the apical footprint is strongly biased to become the neuronal daughter rather than a progenitor. Second, the non-apical daughter can repolarize to form an apical domain and replenish the apical progenitor pool.

Apical proteins are necessary to promote neurogenic fates

Since our results demonstrate that in asymmetrically fated divisions the daughter that inherits the apical footprint is most likely to become the neuron, this suggests that apical proteins could promote neuronal fate. To test this we analysed daughter fates by time-lapse microscopy in embryos in which the function of either Par3 or aPKC had been reduced by antisense morpholino injection. We generated a relatively mild knockdown of these proteins in order not to affect the overall integrity of the neuroepithelium. We were unable to detect a significant difference in the proportion of symmetric proliferative (PP) and neurogenic divisions (NN+NP) observed in wild type embryos or embryos injected with control morpholino. However, a pronounced and significant decrease in neurogenic divisions was observed in embryos injected with either aPKC or Par3 morpholinos (Fig. 7). This supports our observations that in asymmetrically fated divisions the more apical daughter is destined to be a neuron and supports the hypothesis that molecular components of the apical footprint are able to promote neuronal fate.

In asymmetric divisions the progenitor inherits the basal process

Regulation of asymmetric fates may result from the integration of several factors that could include both basal as well as apical information. Some previous data suggests inheritance of the basal process of neural progenitors is important for determining apical progenitor fate²³. However other observations suggest the neuronal daughter frequently inherits the basal process². To evaluate the possible role of inheritance of the basal process by zebrafish neural progenitors we labelled progenitors with membrane-GFP and followed the fate of the basal process during division. As previously reported the basal process often splits along its length prior to cell body cytokinesis²⁴ and is often

asymmetrically inherited (Supplementary Fig. 7, Movie 13). To investigate if basal process inheritance was correlated with asymmetric inheritance of the apical footprint we co-expressed Par3-GFP and membrane-RFP in the same cells and followed the distribution of both the basal process and apical footprint. In divisions with asymmetric Par3 inheritance, inheritance of the basal process is strongly biased to the Par3-GFP-negative daughter (18/20) (Fig. 8a), which our present work suggests is likely to become a progenitor. To examine whether the daughter destined to be the progenitor inherits the basal process in asymmetrically fated divisions, we followed the inheritance of the basal process and the subsequent fate of daughter cells. We observed the basal process can be asymmetrically inherited in both symmetric proliferative and asymmetrically fated divisions. However, in asymmetrically fated divisions the basal process is always inherited by the progenitor daughter (6/6) (blue arrow in Fig. 8b). These results are consistent with the view that in early neurogenesis either inheritance of the basal process itself or the molecular components associated with the basal process can play a role in determining progenitor cell fate during asymmetric divisions.

Discussion

Our work uses live imaging and definitive indicators of fate to analyse the cellular events of asymmetric divisions that generate a neuron and a progenitor at a precise early stage of neurogenesis in a vertebrate. By using the transparent zebrafish we analyse sub-cellular processes and their implication in cell fate decisions for the first time in an intact vertebrate embryo, thus eliminating worries inherent in using *in vitro* systems or interpreting static images from fixed samples. By observing Par3-GFP to outline the apical footprint we provide the first direct evidence that there is a correlation between asymmetric apical inheritance and asymmetric daughter cell fate in a vertebrate neural tube. Contrary to previous predictions we show the daughter that inherits the apical footprint becomes the neuron and the more basal daughter that inherits the basal process replenishes the apical progenitor pool. These observations suggest that during asymmetric division the apical footprint biases cells towards a neuronal fate and this is supported by knockdown experiments that demonstrate reduction of either of the apical

proteins Par3 or aPKC results in a decrease in neurogenic divisions. Inheritance of the apical footprint by neuronal daughters and inheritance of the basal process by the progenitor daughter suggest that asymmetric fate decisions may require integration of both apical and basal information (Supplementary Fig. 8).

Although our results demonstrate asymmetric inheritance of the apical footprint strongly correlates with asymmetric daughter fates, the data shows this is not an all or none process as we also observe symmetric Par3-GFP in 20% of asymmetrically fated divisions (NP) and asymmetric Par3-GFP in 25% of symmetric proliferative divisions (PP). One likely reason for these observations is that symmetric and asymmetric fates in progenitors probably result from integrating inherited information in apical and basal domains with several other factors such as Delta/Notch signalling²⁵, interkinetic nuclear migration^{26,27,28} or cell cycle length^{20,29,30} all of which may influence fate decisions. It is also possible that the neural tube consists of a mixture of progenitors some with neurogenic potential and others without neurogenic potential^{6,31}; in this case progenitors with no neurogenic potential will never generate a neuronal daughter no matter how the apical domain is inherited. Therefore we suggest that asymmetric inheritance of apical and/or basal domains predisposes but does not irreversibly commit daughters to adopt asymmetric fates.

Our data contradicts previous predictions that during asymmetric divisions the more apical daughter cell should become the progenitor. Since the daughter that inherits the apical domain is likely to inherit the apical belt of adherens junctions as well as the apical membrane^{32,33} the simple prediction would be that this apical daughter would remain fixed into the neuroepithelium as a progenitor and the daughter that does not receive the junctional belt would be free to escape the epithelial organisation and differentiate as a neuron^{14,16}. A recent analysis of the constituent components of the junctional belt of cortical progenitors suggest that in fact the Par3/aPKC domain may be asymmetrically inherited while a larger junctional domain that includes both ZO-1 and N-cadherin immunoreactivity is shared between the two daughters¹⁷. This result suggested that both daughters in asymmetric divisions may inherit the adhesive components of adherens junctions (and thus remain at least temporarily attached to the apical surface) while only one daughter inherits the aPKC/Par3 domain and true apical membrane. If the adhesive

components of the zebrafish adherens junctions also lie more basal than the Par3-GFP domain then this could explain how the Par3-GFP negative daughter is able to remain close to the apical surface to rebuild an apical footprint as it replenishes the progenitor pool. However in divisions with cleavage planes close to parallel to the apical surface (for example Figures 6a and Movie 11) we observe that even basal daughters with no apparent contact with the apical surface are able to generate a process that grows to establish a new contact with the apical surface. It seems unlikely in this circumstance that the basal daughter inherits adherens junctions and this suggests that apical attachment can be achieved by regenerating new adherens junctions without inheritance of the original junctional complex. Although we haven't monitored adherens junctions directly, our time-lapse imaging does reveal rapid repolarization of Par3 fusion proteins in the more basal daughters that do not inherit the original Par3 domain. Live imaging may not be able to resolve very small cellular processes that could maintain some apical contact even from the basal daughters of parallel cleavages, thus in future studies it will be important to determine exactly which molecular and structural components are required to be inherited to ensure one daughter replenishes the apical progenitor pool.

The ability to retain or regain attachment to the apical surface is crucial to maintain the apical progenitor population and appears to be lost in mouse cortical slices²³ and chick spinal cord *in ovo*⁷ when cleavage planes are experimentally forced away from their normal near vertical orientation. Loss of progenitors from the apical surface to an ectopic position in the mantle layer is also observed following Par3 over-expression in chick embryo spinal cord³³. Although cleavage planes were not measured in that study, we find even mild Par3 over-expression can destabilize cleavage orientation. Thus the loss of apical progenitors in the chick could also result from increased numbers of oblique and parallel cleavage planes that hinder progenitors from re-attaching to the apical surface.

Loss of function studies in our work suggest apical proteins play a role in neuronal fate determination. This is reminiscent of recent examinations of Par3 function in mammary gland epithelia and aPKC function in zebrafish retina that indicated their requirement in progenitor differentiation rather than progenitor maintenance^{27,34}. However, in mouse cortex, Par3 loss of function increases the proportion of early born neurons and suggests that Par3 is required to maintain apical progenitors in a proliferative state^{11,13}. This

appears difficult to reconcile with our present results that show that the neuronal daughter in asymmetric divisions inherits Par3 and that reduction of Par3 function leads to a loss of neurogenic divisions. While there could be temporal, regional or species specific roles for Par3 we believe our results may be compatible with loss of apical progenitors because Par3 function may be necessary for the more basal daughter to re-establish apical contacts. Thus loss of Par3 would lead to ectopic progenitor divisions seen in the cortical studies¹³. Precise comparisons between our results and those in the mammalian cortex will be difficult until either behaviour of individual cells in the developing cortex is examined or the function of apical proteins is more precisely addressed in zebrafish neural tube.

Our work suggests that co-ordinated and complementary inheritance of apical footprint and basal domains may be required for asymmetric fates (NP). Our current knowledge about function of apicobasal polarity proteins and the signals contained in the basal process in vertebrates, does not allow us to explain how their asymmetries might influence asymmetric fates or why it is the prospective neuron that inherits the apical complex. One possibility is asymmetric inheritance will regulate the Notch signalling pathway^{13,35} as this is a powerful determinant of cell fate. Endocytosis of Notch pathway components is required to activate and modulate this pathway^{36,37} and polarity proteins have been shown to regulate^{16,38,39} and be regulated by endocytic events⁴⁰. Together with our own data this raises the possibility that in the vertebrate neural tube asymmetric inheritance of apical and basal components may influence Notch endocytosis and bias Notch pathway activation in the two daughter cells to promote asymmetric fates.

Authors contributions

PA and JC conceived the project, planned the experiments and wrote the manuscript. PA generated and analysed most of the data and built the Par3-RFP construct. AR built the Par3-GFP and Numb-GFP constructs and generated data on Numb. DB analysed the size distribution of the apical end feet (Supplementary Figure 1). EB did the statistical analysis.

Acknowledgments

We thank Claudio Araya, Clare Buckley, Kara Cervený, Gemma Girdler, Andrew Lumsden, Marion Wassef and Xiaoyun Ren for comments on the manuscript, and the UCL and KCL fish facilities for providing and taking care of fish. We thank Fahad Mahmood for the aPKC immuno staining. PA was supported by an EMBO fellowship (ALTF-764-2005) and EU Marie Curie fellowship (IEF 039695).

Figure legends

Figure 1 Live imaging reveals symmetric and asymmetric daughter fates.

Seventeen selected frames from Movie 1, a 9 hour time-lapse sequence of two nearly adjacent progenitors expressing membrane-GFP and H2B-RFP. The cell on the left undergoes an asymmetrically fated division generating a neuron and an apical progenitor (NP), and the right hand cell undergoes a symmetrically fated division generating two apical progenitors (PP). Neuronal fate is identified by the expression of HuC:GFP in both the cytoplasm and nucleus (the nucleus thus becomes yellow, white arrow). Before the onset of HuC:GFP expression the neuronal daughter detaches from the apical surface (white asterisk in timepoint 1h24min). Progenitor fate is identified by a subsequent division (blue arrows). Lineage trees for the two original cells are illustrated in last panel. Apical surface is outlined by dotted line. Scale bar: 12µm.

Figure 2 Par3-GFP outlines the apical footprint of neural progenitors.

(a) Projected live image from confocal stack shows Par3-GFP is strongly polarized to the apical end of two adjacent neural progenitors. Distinct rings of expression outline the apical domain of the endfeet. A low level of cytoplasmic GFP expression is also apparent. Scale bar: 10µm.

(b) Projected live image from confocal stack shows that a single progenitor entering mitosis with metaphase plate oriented perpendicular to the apical surface retains apical ring of Par3-GFP. The Par3 ring appears to disappear behind the metaphase plate in this projected view. Scale bar: 5µm.

(c) Projection from confocal stack of whole mount brain viewed from ventricular surface reveals Par3-GFP is co-expressed with the apical ring of the junctional protein ZO-1

(red).

(d) Frozen section reveals intense spots of Par3-GFP are co-expressed with the most intense domain of aPKC (red).

(e) Projection from confocal stack of whole mount brain viewed from ventricular surface reveals Par3-GFP is co-expressed with the apical ring of filamentous actin revealed by the expression of F-actin-Cherry (red). Scale bar for (c) (d) and (e): 5 μ m.

Figure 3 Par3-GFP can be symmetrically or asymmetrically inherited during cell division.

(a) Time-lapse sequence from Movie 2 of progenitor division with symmetric inheritance of Par3-GFP to both daughters.

(b) Time-lapse sequence from Movie 3 of progenitor division with asymmetric inheritance of Par3-GFP to only one of the two daughters.

(c) Confocal image to show asymmetric inheritance of Par-GFP is matched by asymmetric inheritance of the endogenous ZO-1 antigen. Apical surfaces are shown by dotted lines and arrow indicates central canal between them. The complete sequence of z-stacks through this dividing cell is shown in Supplementary Fig. 3.

(d) Single frame from time-lapse sequence showing asymmetric inheritance of Par3-GFP despite a near perpendicular cleavage angle (α is measured between the cleavage plane and the plane of the apical surface. Scale bars for all: 10 μ m.

Figure 4 Asymmetric inheritance of Par3-GFP correlates with asymmetric daughter fate.

(a) Time lapse sequence from Movie 7 showing asymmetric inheritance of Par3-GFP in a division with near parallel cleavage (the complete sequence of z-stacks through this dividing cell is shown in Supplementary Fig. 6a). The apical daughter (white arrowed) inherits Par3-GFP and migrates into the mantle zone and begins to express the neuronal protein HuC:GFP at timepoint 8h03min. The HuC:GFP expression becomes more obvious at timepoint 9h52min as the nucleus turns orange, as shown by the merged and non merged images of a single z-plane. The non-apical daughter that does not inherit Par3-GFP (blue asterisk) undergoes a cycle of interkinetic nuclear migration before

dividing at timepoint 8h18min (blue arrow). White asterisk shows the apical detachment of neuronal daughter. Apical surface is shown by dotted lines. Scale bar: 5µm.

(b) Quantification of 47 divisions reveals that asymmetrically fated divisions (NP) are much more likely to distribute Par3-GFP asymmetrically than symmetrically fated divisions (PP). The correlation between fate and inheritance is statistically significant however not absolute. *Chi square test, $p=0.001$.

Figure 5 Par3-GFP weakly destabilises cleavage orientation.

(a) and (b) Quantification of 125 divisions shows that although most divisions in both membrane-GFP and Par3-GFP expressing cells occur with a cleavage within 15° of perpendicular, the expression of Par3-GFP does significantly increase the number of more oblique cleavages. We compared the number of near perpendicular cleavages (75-90°) with cleavages of less than 75° in wild-type divisions ($n=47$ divisions) and in Par3-GFP expressing cells ($n=78$), *Chi square test: $p<0.01$. In a subset of the Par3-GFP expressing cells we were able to both measure cleavage angle and follow daughter cell fate to determine the relationship between symmetrically (PP and NN) and asymmetrically (NP) fated divisions and cleavage angle. These divisions are shown by the blue and red dots.

(c) Quantification of the fate of 25 membrane-GFP expressing progenitors and 58 Par3-GFP expressing progenitors reveals Par3-GFP does not significantly alter the ratio of symmetric proliferative (PP) and asymmetrically fated divisions (NP). A small number (4/58) of additional symmetric neurogenic divisions (NN) are generated from the Par3-GFP expressing progenitors.

Figure 6 Non-apical daughters replenish the progenitor pool.

(a) Projection of 2 z-planes of a progenitor labelled with membrane-GFP and dividing with a parallel cleavage. The non-apical daughter (blue asterisk) appears to be initially disconnected from the apical surface immediately after mitosis but begins to make a visible process aimed at the apical surface just 3 minutes after mitosis and this process extends further to reach the apical surface 21 minutes later. This daughter divides at the apical surface 12 hours later (blue arrows). The elongation of the basal process towards

the basal surface by the more apical daughter cell can be seen at time points 21min and 22min30sec and resembles to a growth cone. Apical surface is shown by the dotted lines. (b) After progenitor division, at the 6 minute time point, the left hand daughter (white asterisk) does not contain a bright Par3-RFP domain (white arrow) compared to its sister cell on the right (white asterisk and white arrowhead). However by the 15 minute time point the left hand daughter has polarized to form a bright apical Par3-RFP domain. The cells of interest are outlined by dotted lines. Images contain another progenitor (yellow asterisk) that does not divide in this period but does have a bright Par3-RFP domain. Three-D reconstructions of both time points are shown in Movie 12. Scale bars: 10 μ m.

Figure 7 The apical proteins Par3 and aPKC are required for neurogenic divisions (NN+NP) in the early neural tube.

Daughter cell fate was assessed by time-lapse analyses in wt embryos and embryos injected with control morpholino or morpholinos against either Par3 or aPKC. Reduction of either Par3 or aPKC resulted in a significant decrease of neurogenic divisions (NP+NN) when compared to control morpholino. *Chi square test: $p < 0.0001$, **Chi square test: $p < 0.001$.

Figure 8 Basal process is inherited by the daughter fated to be the progenitor in asymmetrically fated divisions.

(a) Basal process is inherited by the Par3-GFP negative daughter. Red arrows indicate thin basal process attached to only one daughter immediately after cytokinesis. At time point 0min the process is seen attached to a small membrane bleb on the surface of the more basal daughter. By time point 4min the membrane bleb has reintegrated into the smooth surface of the cell and the attachment of the basal process to this daughter is clear. The more apical daughter sits at the apical surface (dotted line) in contact with the central canal. A third cell labelled with black asterisk is in the same frame as the two daughter cells of interest but it does not divide.

Bottom panels in (a) include green channel to reveal Par3-GFP distribution. This is only visible in the more apical daughter (white arrow), not in the daughter inheriting the basal process.

(b) The daughter inheriting basal process becomes the progenitor in asymmetrically fated divisions. In the top panel, time point 0min shows the basal process splits (red arrow) before cell body cytokinesis takes place. By 7min, small z projection in grey reveals cytokinesis has occurred, the daughter on left (blue arrow) inherits a bifurcated basal process (red arrows), while right hand daughter (white arrowed) does not. The last two micrographs show two distinct z-levels of the daughter cells just following division: the daughter inheriting the basal process (z0, blue arrow) and the daughter that does not inherit the basal process (white arrow) are 4 μm apart in the z plane. In the middle panels, the daughter cell that inherited the basal process divided later at time point 17h41min (blue arrow) proving it is a progenitor. In the bottom panels, by time point 24h00min the daughter that did not inherit the basal process has differentiated into a commissural neuron with clear axonal process crossing the ventral midline. A diagrammatic transverse section is also shown to illustrate neuronal morphology. All scale bars: 10 μm .

Methods

mRNA injections

Plasmids containing the DNA coding for H2B-RFP, membrane-GFP, Par3-GFP¹⁹, Par3-RFP and Numb-GFP⁴², F-actin-Cherry (UTR-actin-Cherry, gift from WA Harris lab) were linearised and the mRNAs synthesised using SP6 mMessenger mMachine kit (Ambion). Single cells were injected at the 64-128 cell stage with the mRNAs of interest to obtain mosaic expression.

Time-lapse imaging in zebrafish

To follow cell fate and neuronal differentiation we used the transgenic zebrafish line tg(HuC:GFP) that express GFP in all neurons⁴³. In other time-lapse experiments we used wild-type embryos. Embryos were anaesthetized with MS222 (Sigma) at 21 to 24hpf and mounted in 1.5% low-melting agarose (Sigma) in a small microscope slide chamber with the dorsal aspect of posterior hindbrain and spinal cord facing up. Images were obtained using long-working distance x40 or x63 water immersion objectives. During the time-

lapse the embryos were maintained at 28.5°C in anaesthetic and 0.003% 1-phenyl-2-thiourea to prevent pigmentation. A series of z-stacks (planes between 1 and 2 µm apart) were obtained every 1-10 minutes for between 1h and 36h depending on the experiment. Data sets were analysed using ImageJ, Volocity 4D analysis software and Fluorender (<http://www.sci.utah.edu/software.html>).

Immunohistochemistry

Antigen localisation was performed on frozen sections or whole-mount embryos. The embryos were fixed at 24hpf in 4% PFA for 2h at room temperature and processed using standard procedures. We used antibodies against aPKC (rabbit, Santa Cruz, diluted 1:500), ZO-1 (mouse, Invitrogen, diluted 1:500) and anti-GFP (chick, Abcam, diluted 1:1000) diluted in PBTween 0.25% 10% serum.

Morpholinos

Par3 and aPKC loss of function were achieved by injection of previously characterised morpholinos^{44,45}. We injected 0.21pmol of Par3 morpholino and 0.385pmol of aPKC morpholino at 1 cell stage followed by a second injection of H2B-RFP mRNA in one cell at 68-128 cells stage. As a control we injected a standard control morpholino sold by GeneTools. The time-lapses were set up as described in the time-lapse imaging section.

Statistical analysis

We test for the effect of Par3-GFP inheritance in cell fate determination, the overexpression of Par3-GFP on cell cleavage orientation and the knockdown of apical proteins on cell fate determination by using a Chi Square test and the Yates correction.

All animal procedures were carried out under Home Office Project Licence number PPL 70/6671.

References

1. Chenn, A., McConnell, S.K. Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis. *Cell* **82**, 631-641 (1995).

2. Miyata, T., Kawaguchi, A., Okano, H., Ogawa, M. Asymmetric inheritance of radial glial fibers by cortical neurons. *Neuron* **31**, 727-741 (2001).
3. Noctor, S.C., Flint, A.C., Weissman, T.A., Dammerman, R.S., Kriegstein, A.R. Neurons derived from radial glial cells establish radial units in neocortex. *Nature* **409**, 714-720 (2001).
4. Noctor, S.C., Martínez-Cerdeño, V., Ivic, L., Kriegstein, A.R. Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat Neurosci.* **7**, 136-144 (2004).
5. Cayouette, M., Raff, M. The orientation of cell division influences cell-fate choice in the developing mammalian retina. *Development* **130**, 2329-2339 (2003).
6. Wilcock, A.C., Swedlow, J.R., Storey, K.G. Mitotic spindle orientation distinguishes stem cell and terminal modes of neuron production in early spinal cord. *Development* **134**, 1943-1954 (2007).
7. Morin, X., Jaouen, F., Durbec, P. Control of planar divisions by the G-protein regulator LGN maintains progenitors in the chick neuroepithelium. *Nat Neurosci.* **10**, 1440-1448 (2007).
8. Attardo, A., Calegari, F., Haubensak, W., Wilsch-Bräuninger, M., Huttner, W.B. Live imaging at the onset of cortical neurogenesis reveals differential appearance of the neuronal phenotype in apical versus basal progenitor progeny. *PLoS ONE* **3**, e2388 (2008).
9. Shen, Q., Zhong, W., Jan, Y.N., Temple, S. Asymmetric Numb distribution is critical for asymmetric cell division of mouse cerebral cortical stem cells and neuroblasts. *Development* **129**, 4843-4853 (2002).
10. Cayouette, M., Barres, B.A., Raff, M. Importance of intrinsic mechanisms in cell fate decisions in the developing rat retina. *Neuron* **4**, 897-904 (2003).
11. Costa, M.R., Wen, G., Lepier, A., Schroeder, T., Götz, M. Par-complex proteins promote proliferative progenitor divisions in the developing mouse cerebral cortex. *Development* **135**, 11-22 (2008).
12. Schwamborn, J.C., Berezikov, E., Knoblich, J.A. The TRIM-NHL protein TRIM32 activates microRNAs and prevents self-renewal in mouse neural progenitors. *Cell* **136**, 913-925 (2009).
13. Bultje R.S. *et al.* Mammalian Par3 regulates progenitor cell asymmetric division via notch signaling in the developing neocortex. *Neuron* **63**, 189-202 (2009).
14. Kosodo, Y. *et al.* Asymmetric distribution of the apical plasma membrane during neurogenic divisions of mammalian neuroepithelial cells. *EMBO J.* **23**, 2314-2324

(2004).

15. Petersen, P.H., Zou, K., Krauss, S., Zhong, W. Continuing role for mouse Numb and Numbl in maintaining progenitor cells during cortical neurogenesis. *Nat Neurosci.* **7**, 803-811 (2004).

16. Rasin, M.R. *et al.* Numb and Numbl are required for maintenance of cadherin-based adhesion and polarity of neural progenitors. *Nat Neurosci.* **10**, 819-827 (2007).

17. Marthiens V, ffrench-Constant C. Adherens junction domains are split by asymmetric division of embryonic neural stem cells. *EMBO Rep.* **10**, 515-520 (2009).

18. Huttner, W.B., Brand, M. Asymmetric division and polarity of neuroepithelial cells. *Curr Opin Neurobiol.* **7**, 29-39 (1997).

19. Von Trotha, J.W., Campos-Ortega, J.A., Reugels, A.M. Apical localization of ASIP/PAR-3:EGFP in zebrafish neuroepithelial cells involves the oligomerization domain CR1, the PDZ domains, and the C-terminal portion of the protein. *Dev Dyn.* **235**, 967-977 (2006).

20. Götz, M., Huttner, W.B. The cell biology of neurogenesis. *Nat Rev Mol Cell Biol.* **6**, 777-788 (2005). Review.

21. Sanada, K., Tsai, L.H. G protein betagamma subunits and AGS3 control spindle orientation and asymmetric cell fate of cerebral cortical progenitors. *Cell* **122**, 119-131 (2005).

22. Zigman, M., Mammalian inscuteable regulates spindle orientation and cell fate in the developing retina. *Neuron* **48**, 539-545 (2005).

23. Konno, D. *et al.* Neuroepithelial progenitors undergo LGN-dependent planar divisions to maintain self-renewability during mammalian neurogenesis. *Nat Cell Biol.* **10**, 93-101 (2008).

24. Kosodo, Y. *et al.* Cytokinesis of neuroepithelial cells can divide their basal process before anaphase. *EMBO J.* **27**, 3151-63.

25. Henrique, D. *et al.* Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina. *Curr Biol.* **7**, 661-670 (1997).

26. Xie, Z. *et al.* Cep120 and TACCs control interkinetic nuclear migration and the neural progenitor pool. *Neuron* **56**, 79-93 (2007). 17. Marthiens, V., Ffrench-Constant, C. Adherens junction domains are split by asymmetric division of embryonic neural stem cells. *EMBO Rep.* **10**, 515-520 (2009).

27. Baye, L.M., Link, B.A. Interkinetic nuclear migration and the selection of neurogenic

- cell divisions during vertebrate retinogenesis. *J Neurosci.* **27**, 10143-10152 (2007).
28. Del Bene, F., Wehman, A.M., Link, B.A., Baier, H. Regulation of neurogenesis by interkinetic nuclear migration through an apical-basal notch gradient. *Cell* **134**, 1055-1065 (2008).
 29. Hodge, R.D., D'Ercole, A.J., O'Kusky, J.R. Insulin-like growth factor-I accelerates the cell cycle by decreasing G1 phase length and increases cell cycle reentry in the embryonic cerebral cortex. *J Neurosci.* **24**, 10201-10210 (2004).
 30. Calegari, F., Haubensak, W., Haffner, C., Huttner, W.B. Selective lengthening of the cell cycle in the neurogenic subpopulation of neural progenitor cells during mouse brain development. *J Neurosci.* **25**, 6533-6538 (2005).
 31. Iacopetti, P. *et al.* Expression of the antiproliferative gene TIS21 at the onset of neurogenesis identifies single neuroepithelial cells that switch from proliferative to neuron-generating division. *Proc Natl Acad Sci U S A.* **96**, 4639-4644 (1999).
 32. Manabe, N. *et al.* Association of ASIP/mPAR-3 with adherens junctions of mouse neuroepithelial cells. *Dev Dyn.* **225**, 61-69 (2002).
 33. Afonso, C., Henrique, D. PAR3 acts as a molecular organizer to define the apical domain of chick neuroepithelial cells. *J Cell Sci.* **119**, 4293-4304 (2006).
 34. McCaffrey, L.M., Macara, I.G. The Par3/aPKC interaction is essential for end bud remodeling and progenitor differentiation during mammary gland morphogenesis. *Genes Dev.* **23**, 1450-1460 (2009).
 35. Langevin, J. *et al.* Lethal giant larvae controls the localization of notch-signaling regulators Numb, Neuralized, and Sanpodo in *Drosophila* sensory-organ precursor cells. *Curr. Biol.* **15**, 955-962 (2005).
 36. Le Borgne, R. Regulation of Notch signalling by endocytosis and endosomal sorting. *Curr Opin Cell Biol.* **18**, 213-222 (2006). Review.
 37. Coumailleau, F., Fürthauer, M., Knoblich, J.A., González-Gaitán, M. Directional Delta and Notch trafficking in Sara endosomes during asymmetric cell division. *Nature* **458**, 1051-1055 (2009).
 38. Georgiou, M., Marinari, E., Burden, J., Baum, B. Cdc42, Par6, and aPKC regulate Arp2/3-mediated endocytosis to control local adherens junction stability. *Curr Biol.* **18**, 1631-1638 (2008).
 39. Leibfried, A., Fricke, R., Morgan, M.J., Bogdan, S., Bellaiche, Y. *Drosophila* Cip4 and WASp define a branch of the Cdc42-Par6-aPKC pathway regulating E-cadherin endocytosis. *Curr Biol.* **18**, 1639-1648 (2008).

40. Nakayama, Y. *et al.* Dynamin participates in the maintenance of anterior polarity in the *Caenorhabditis elegans* embryo. *Dev Cell* **16**, 889-900 (2009).
41. Tawk, M. *et al.* A mirror-symmetric cell division that orchestrates neuroepithelial morphogenesis. *Nature* **446**, 797-800 (2007).
42. Reugels, A.M., Boggetti, B., Scheer, N., Campos-Ortega, J.A. Asymmetric localization of Numb:EGFP in dividing neuroepithelial cells during neurulation in *Danio rerio*. *Dev Dyn.* **235**, 934-948 (2006).
43. Park, H.C. *et al.* Analysis of upstream elements in the HuC promoter leads to the establishment of transgenic zebrafish with fluorescent neurons. *Dev Biol* **227**, 279–293 (2000).
44. Horne-Badovinac, S., *et al.* Positional cloning of heart and soul reveals multiple roles for PKC lambda in zebrafish organogenesis. *Curr Biol.* **11**, 1492-1502 (2001).
45. Wei, X. *et al.* The zebrafish *Pard3* ortholog is required for separation of the eye fields and retinal lamination. *Dev Biol.* **269**, 286-301 (2004).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8