A nutrient-sensitive restriction point is active during retinal progenitor cell differentiation

Nicola K. Love¹, Nandaki Keshavan¹, Rebecca Lewis¹, William A. Harris¹,* and Michalis Agathocleous¹,2

ABSTRACT
In many growing tissues, slowly dividing stem cells give rise to rapidly proliferating progenitors that eventually exit the cell cycle and differentiate. Growth rates are limited by nutrient availability, but it is unclear which steps of the proliferation-differentiation programme are particularly sensitive to fuel supplies. We examined how nutrient deprivation (ND) affects stem and progenitor cells in the ciliary marginal zone (CMZ) of the amphibian retina, a well-characterised neurogenic niche. We show that ND specifically blocks the proliferation and differentiation of progenitor cells through an mTOR-mediated mechanism. By contrast, the identity and proliferation of retinal stem cells are insensitive to ND and mTOR inhibition. Refeeding starved retinas in vitro rescues both proliferation and differentiation, and activation of mTOR is sufficient to stimulate differentiation even in ND retinas. These results suggest that an mTOR-mediated restriction point operates in vivo to couple nutrient abundance to the proliferation and differentiation programme in retinal progenitor cells.

KEY WORDS: Differentiation, mTOR, Nutrient deprivation, Proliferation, Restriction point, Retina, Xenopus laevis

INTRODUCTION
Adequate nutrition is vital for growth (Hietakangas and Cohen, 2009), and nutrient deprivation (ND) has been implicated in disease (Jackson, 2002) and aging (Jasper and Jones, 2010). Despite the fact that growth control is exercised through the precise regulation of stem and progenitor cell lineages, little is known about how this regulation is affected by impaired nutrition.

Studies in Drosophila show that ND slows cell proliferation (Drummond-Barbosa and Spradling, 2001; McLeod et al., 2010) and that feeding induces quiescent neuroblasts to re-enter the cell cycle (Chell and Brand, 2010; Sousa-Nunes et al., 2011). Although cell cycle control of proliferating populations operates in tandem with the control of differentiation, it is not fully understood how the latter is affected by nutrient availability. In Drosophila, ND promotes stem and progenitor cell differentiation in the germline (Hsu and Drummond-Barbosa, 2009) and the lymph gland (Benmimoun et al., 2012; Shim et al., 2012). If stem and progenitor cells deprived of nutrients are forced to differentiate without dividing, tissues could become depleted of stem cells and thus be unable to grow even if food becomes available again. However, in most systems it seems that there are mechanisms in place to allow the re-initiation of growth with the return of nutrient availability, suggesting that a particular step in the proliferation-differentiation programme might be sensitive to nutrient loss, so that stem cells are maintained even if other cells are lost.

To investigate the effects of ND on the proliferation-differentiation programme, we used amphibian and fish retinas, which grow throughout life by the addition of new cells from the ciliary marginal zone (CMZ), a neurogenic niche at the retina periphery (supplementary material Fig. S1A) (reviewed by Agathocleous and Harris, 2009). Within the CMZ, slowly dividing retinal stem cells reside at the extreme periphery (supplementary material Fig. S1B,C) (Xue and Harris, 2012). Just central to these cells are rapidly proliferating retinal progenitor cells that express markers of neuronal commitment, and at the central edge of the CMZ are cells that are poised to exit the cell cycle and terminally differentiate into neurons and glial cells (Perron et al., 1998; Xue and Harris, 2012) (supplementary material Fig. S1B,C). The CMZ thus recapitulates in space from periphery to centre the temporal sequence of development from uncommitted stem cells, to committed rapidly dividing progenitors, to differentiating neurons (Harris and Perron, 1998). Several studies have elucidated the transcriptional and signalling mechanisms behind this differentiation cascade in the retina (Borday et al., 2012; Cerveny et al., 2010; Denayer et al., 2008; Wehman et al., 2005; El Yakoubi et al., 2012). Here, we examine how nutrient availability regulates the proliferation-differentiation programme in the CMZ. We find that ND induces an mTOR-dependent block in proliferation and differentiation specifically in progenitor but not stem cells, defining a nutrient-sensitive restriction point that operates in the differentiation programme of retinal stem and progenitor cells.

RESULTS
Nutrient deprivation causes a decrease in CMZ proliferation
We deprived Xenopus laevis larvae of their physiological internal nutrients (Jorgensen, 2008; Jorgensen et al., 2009) by dissection of the yolk deposits in the gut, starting at stage 34/35 (Fig. 1A), ~36 hours post-fertilisation (hpf). From this stage onwards, cells in the central retina are mostly postmitotic, with proliferating cells confined to the CMZ (Holt et al., 1988). These ND embryos survived well and 2 days later, at stage 41, were morphologically normal (Fig. 1B,C), although smaller than controls with proportionally smaller eyes (Fig. 1D-F). The CMZs of ND larvae were smaller and had fewer cells than controls (Fig. 1E,F). ND caused a significant drop in incorporation of the nucleotide analogue EdU in the CMZ (Fig. 1G,H). There was no conspicuous increase in CMZ cell apoptosis (supplementary material Fig. S2A-D). These results suggest that ND prevents proliferation in the CMZ. The loss of proliferation was due to ND rather than injury
to the embryo, as no reduction in proliferation was observed when less than a quarter of the yolk was removed (supplementary material Fig. S3).

To test whether the effects of ND on the CMZ were specific to *Xenopus*, we also deprived zebrafish larvae of nutrients by removing the majority of their yolk at 56 hpf. Both the number of CMZ cells (supplementary material Fig. S4A) and CMZ proliferation (supplementary material Fig. S4B,C) were decreased in ND fish at 84 and 112 hpf. CMZ proliferation is therefore dependent on abundant nutrients in both fish and frogs.

Detection of cell cycle gene expression by *in situ* hybridisation showed that ND inhibited the expression levels of *cyclin A2* and *cyclin D1*. ND did not significantly change the expression levels of the cyclin-dependent kinase inhibitor p27Xic1, although it appeared to expand its expression domain (Fig. I; supplementary material Fig. S5A). These data indicate that the reduction in CMZ proliferation after ND might be ultimately mediated by changes in the expression of specific cell cycle regulators.

**The reduction in CMZ proliferation is due to a specific effect on retinal progenitor cells**

To test whether all CMZ cells are similarly impaired in proliferation by nutrient withdrawal, or whether stem and progenitor cells respond differently, we measured EdU incorporation in cells positive for the stem cell marker Hes1 (M. Perron, personal communication). We saw a significant decline in the proliferation of EdU-positive progenitors in the Hes1-positive stem cell population. n=3, 12 embryos/condition. (G,J) Error bars show 95% confidence intervals; ***P<0.001 (unpaired t-test); ns, not significant. Scale bars: 100 μm (E,F); 50 μm (H,I).

Nutrients are necessary for the acquisition of the committed neural progenitor fate but not for the maintenance of the most immature stem/progenitor cells

We next investigated whether the reduced size and cell number of the CMZ after ND in the absence of cell death is solely due to changes in proliferation or whether impairments in the...
differentiation programme are also involved. Early stage progenitor cells express n-Myc (Xue and Harris, 2012), Sox2 (Agathocleous et al., 2009; Van Raay et al., 2005) and several proneural basic helix-loop-helix (bHLH) genes (Perron et al., 1998). These transcription factors define the progenitor fate and are necessary for neurogenic competence. We assayed the CMZ for their expression by in situ hybridisation. The domain of expression of these progenitor genes was reduced, suggesting fewer progenitors, in agreement with our data for a smaller CMZ and specific deficits in progenitor proliferation (Fig. 2A,B; supplementary material Fig. S5B), Crucially, however, we also found that the levels of expression of the bHLH genes ascl1, atoh7, neurogenin2, neuroD and ath3 and of sox2 and n-Myc were all strongly reduced by ND, as judged by the intensity of in situ hybridisation staining (Fig. 2A,B; supplementary material Fig. S5B; see Fig. 6A,B for quantification). As these genes are crucial for differentiation, these findings suggest that, unlike the progenitors, the stem cells in the extreme periphery of the CMZ are relatively immune to ND.

Committed progenitor cells continue to differentiate normally following ND

Retinal progenitors in the more central CMZ express proneural genes, committing them to differentiation (Harris and Perron, 1998; Perron et al., 1998). In ND retinas, these progenitors are lost, indicating that their production ceases. But what happens to the progenitors that were already present at the start of ND? Do they complete their differentiation programme even in the absence of continued proliferation?

To answer this question, embryos were exposed to EdU for just 20 minutes, and then washed and nutrient deprived. The fate of these EdU-positive cells was examined 3 days later. As would be expected in normal retinas, we found EdU cells located in the differentiated layers of the retina (Fig. 3A). However, and perhaps surprisingly, ND retinas showed significantly more EdU-positive terminally differentiated cells outside of the CMZ than normal embryos, suggesting that many progenitors quickly exited the cell cycle and differentiated after ND (Fig. 3B). Some of these EdU-positive cells were labelled with Islet1, a marker of terminal differentiation of
CMZ proliferation (Fig. 4A,B), demonstrating functionally that ND but no serum or growth factors, resulted in a significant increase in retinas from ND embryos ex vivo from ND animals into nutrient-rich media. We found that culturing intestinal tracts. To circumvent this problem, we explanted whole eyes following ND.

Re-feeding promotes reconstitution of the CMZ

If retinal stem cells are maintained during ND, then feeding a starved embryo may re-populate the CMZ. Unfortunately, ND by dissection of yolk prevents feeding in vivo, as the experimental animals lack intestinal tracts. To circumvent this problem, we explanted whole eyes from ND animals into nutrient-rich media. We found that culturing retinas from ND embryos ex vivo in L15 medium, containing nutrients but no serum or growth factors, resulted in a significant increase in CMZ proliferation (Fig. 4A,B), demonstrating functionally that ND CMZ cells are not lost and are still responsive to growth-promoting conditions. Decreasing nutrient levels from 60% to 30% L15 for a 2-hour period decreased CMZ proliferation acutely in a dose-dependent manner (Fig. 4C), suggesting that proliferation is dynamically, and rapidly, attenuated by nutrient availability.

We next asked how quickly re-feeding initiates the repopulation of the CMZ. Remarkably, in as little as 20 minutes after explanting eyes into L15, a burst of EdU uptake could be observed in the peripheral edge of the CMZ (Fig. 4D), and by 2 hours there was a large increase in EdU uptake in the CMZ compared with ND retinas (Fig. 4E). After 24 hours, re-fed retinas showed increased expression of the cell cycle gene cyclin D1 and a significant upregulation of the proneural genes ascl1 and atoh7 (Fig. 4F,G; supplementary material Fig. S6A). These results demonstrate that the retinal stem cells at the CMZ periphery, and the progenitors whose proliferation had been blocked by ND, are still responsive and, with a new supply of nutrients, are able to reinitiate proliferation and differentiation to reconstitute the entire CMZ.

The mTOR pathway is involved in the nutrient-sensitive restriction point

The serine/threonine kinase mTOR regulates cell growth, proliferation and survival by sensing and responding to nutrient availability (Sengupta et al., 2010; Wang and Proud, 2009) and is involved in the regulation of Drosophila eye development (Lanet et al., 2013; McNeill et al., 2008). To test whether mTOR regulates the effects of ND on the CMZ, we first immunostained for phospho-Ribosomal protein S6 (pS6), the cardinal marker of mTOR activity. pS6 was mostly absent from the differentiated cells in the retina but stained the CMZ intensely (Fig. 5A). Within the CMZ, it marked the progenitor cells, but not the most peripheral undifferentiated stem cells (Fig. 5B). pS6 staining was reduced by ND to levels similar to those of embryos treated with the mTOR inhibitor rapamycin (Fig. 5A,B). Thus, in CMZ progenitors, as in other systems, nutrients are necessary for mTOR activity.

We next asked whether mTOR is necessary for CMZ proliferation. Incubating embryos with rapamycin, like ND, decreased eye growth (Fig. 5C) and inhibited proliferation in the CMZ (Fig. 5D). Both rapamycin treatment and ND reduced the fraction of the G2/M population that was EdU positive after a 1.5-hour pulse, suggesting a G2/M slowdown (Fig. 5E,F). Also similarly to ND, rapamycin inhibited expression of Cyclin D1 and Cyclin A2 (Fig. 5G), without significantly changing the level of p27Xic1 expression (Fig. 5G). These results suggest that mTOR signalling is sensitive to nutrient levels and is necessary for cell proliferation in the CMZ.

Rapamycin caused changes in gene expression similar to those observed with ND. The intensity of expression of the proneural genes ascl1, atoh7, ath3 and neurogenin2 in the central CMZ was significantly decreased both by ND and rapamycin treatment (Fig. 6A). Rapamycin also reduced the expression levels of the central progenitor markers sox2 and n-Myc, which lie upstream of proneural genes in the differentiation cascade (Van Raay et al., 2005; Xue and Harris, 2012) (Fig. 6B), but did not attenuate the expression of rx1 (Fig. 6C), c-Myc (Fig. 6D), hes1 or hes4 (Fig. 6E), as expressed by the most peripheral CMZ stem cells.

These results show that blocking mTOR activity mimics many of the effects of ND in the CMZ, raising the possibility that mTOR mediates the effects of ND. This hypothesis predicts that stimulation of the mTOR pathway in the absence of nutrients might rescue the ND phenotype. mTOR can be activated by the insulin mimetic compound and PTEN inhibitor bpV(phen) (hereafter bpV), which promotes PI3K signalling (Posner et al., 1994; Schmid et al., 2004).

Fig. 3. Committed progenitor cells continue to differentiate normally following ND. (A) Many cells labelled with EdU 20 minutes prior to ND were found in the differentiated central retina after a 3-day chase period. n=3, 12 embryos/condition. (B) These cells increased in number in the ND condition compared with control retinas. n=3, 12 embryos/condition. Error bars show 95% confidence intervals; *P<0.05 (unpaired t-test). (C, D) High magnification of the central retina, which contains differentiated cells. Some EdU-labelled cells were also positive for the neuronal marker Islet1, which marks specific subsets of amacrine and bipolar cells in the inner nuclear layer of the differentiated retina. n=3, 12 embryos/condition. Scale bars: 40 μm (C); 10 μm (D).
When we treated ND larvae with bpV, we found a robust rescue of pS6 specifically in the CMZ (Fig. 7A,B), similar to what was observed in explant cultures upon re-feeding (supplementary material Fig. S6B). Activation of mTOR by bpV in ND embryos was also sufficient to rescue n-Myc and sox2 expression in the central CMZ (Fig. 7C,F) and the expression of the proneural genes ascl1 and atoh7 (Fig. 7D,F). Interestingly, bpV did not cause an upregulation of pS6 in the CMZ stem cell zone at the far periphery, which also maintained expression of hes1 and hes4 (Fig. 7E), consistent with the restoration of mTOR signalling only in its physiological spatial domain. Treatment of control embryos with bpV did not result in an upregulation of pS6, nor did it have an influence on progenitor proliferation or differentiation (supplementary material Fig. S7A-E).
Although bpV rescued the differentiation block after ND, it failed to rescue the decrease in eye size (Fig. 8A) and proliferation (Fig. 8B) caused by ND. Consequently, the block by ND on progenitor differentiation can be uncoupled from, and is not simply downstream of, the impairment in progenitor proliferation. It is interesting to note in this regard that retinas from ND animals do show a transient increase in EdU incorporation 2 hours after the addition of bpV (Fig. 8D,E), and a partial rescue of Cyclin D1 expression (Fig. 8C). One possibility is that mTOR activation in the absence of nutrients does attempt to turn on the proliferation programme but, despite mTOR being necessary for progenitor proliferation (Fig. 5), it is insufficient to drive proliferation in the absence of actual nutrients. Indeed, at 24 hours of bpV treatment, proliferation receded to the levels observed in ND embryos (Fig. 8D,E). Therefore, whereas the effects of ND on differentiation are mediated by mTOR, its effects on proliferation are likely to be mediated by additional cellular mechanisms.

These results support the idea that mTOR signalling, which is lost as a result of ND, is essential for both the differentiation and proliferation of retinal progenitor cells. Even in the absence of nutrients, mTOR is sufficient to drive the progenitor differentiation programme. Retinal stem cells, by contrast, are not dependent on mTOR or abundant nutrients for their maintenance and proliferation.

**DISCUSSION**

We introduce a new ND paradigm for frog and fish embryos and use it to uncover a nutrient-sensitive restriction point in the proliferation-differentiation programme in the CMZ, a well-organised retinal stem cell niche. When embryos are deprived of nutrients, proliferation in the CMZ is shut down and committed progenitors quickly...
differentiate. However, the slowly dividing stem cells at the extreme periphery of the CMZ are maintained throughout ND. Our results indicate that nutrient abundance controls the expression of multiple genes required for a specific step in the proliferation-differentiation programme, defining a restriction point downstream of retinal stem cells. This means that the CMZ can be repopulated and growth can re-initiate as soon as nutrients become available again. Previous work in adult flies and mice showed that some intestinal and germine stem cells are maintained during caloric restriction or amino acid deprivation, whereas differentiated progenitor populations may be more reduced (Drummond-Barbosa and Spradling, 2001; McLeod et al., 2010; Yilmaz et al., 2012). Although differences are expected between the physiology of adult caloric restriction and embryonic ND, the nutrient-sensitive restriction point that we describe here could play a role in other tissues to enable stem cells to preferentially withstand ND compared without losing the capacity to reinitiate growth if conditions of ND are plentiful return.

Key to the response of progenitor cells to nutrient availability is the cellular nutrient-sensing pathway mediated by mTOR. Several growth factors, including the mTOR activator insulin/IGF, promote retinal progenitor proliferation (Agathocleous and Harris, 2009). Activation of mTOR by deletion of PTEN, a negative regulator of the PI3K/mTOR pathway, leads to premature neurogenesis in the developing central retina (Jo et al., 2012), suggesting that the PI3K/mTOR pathway, leads to premature neurogenesis in the central CMZ of ND and rapamycin-treated embryos. n=8, 32 embryos/condition. (D) c-Myc expression levels remained unchanged; however, the domain of c-Myc expression was reduced in the central CMZ of ND and rapamycin-treated embryos. n=8, 32 embryos/condition). (E) Expression levels of the stem cell marker genes hes1 and hes4 remained unchanged (n=4, 16 embryos/condition). In all cases, error bars show 95% confidence intervals; ***P≤0.001, **P≤0.01, *P≤0.05 (unpaired t-test).

In summary, our results support a model in which abundant nutrients activate mTOR causing both proliferation and production of committed retinal progenitor cells. When mTOR is inhibited under conditions of ND, these cells differentiate quickly but are not replenished. The remaining, presumably earlier stage, progenitors stop dividing and their differentiation programme is blocked upstream of the proneural genes. Thus, ND leads to the preferential loss of committed progenitors, but it does not block retinal stem cell fate or proliferation. Our results thus define a nutrient-sensitive restriction point in the proliferation-differentiation programme within retinal progenitor cells in the CMZ, and provide a mechanism whereby growth can be restricted, when nutrients are unavailable, without losing the capacity to reintiate growth if conditions of plenty return.

MATERIALS AND METHODS
Animal maintenance and pharmacological treatment Xenopus laevis embryos, obtained by fertilisation, were raised in 0.1× modified Barth’s solution (MBS) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Larvae were nutrient deprived at stage 34/35 and analysed at stage 41 unless otherwise indicated. For drug treatments, 1-5 μM rapamycin (Calbiochem) or 300 nM bpV(phen) (Calbiochem) were bath applied in embryo medium at stages 28 and 34, respectively, or at stages otherwise indicated. Zebrafish embryos were raised at 25-32°C and staged as hpf (Kimmel et al., 1995). All animal work was approved by Local Ethical Review Committee at the University of Cambridge and performed in accordance with the UK Home Office license PPL 80/2606.
Dissection of yolk from *Xenopus* and zebrafish

*Xenopus* and zebrafish embryos, like other externally developing organisms, rely on maternally derived yolk platelets for nutrition during the first few days of life (Jorgensen et al., 2009). The yolk sacs of stage 34 *Xenopus* embryos were removed by microdissection using fine dissecting pins as illustrated (Fig. 1A). Removal of yolk from larval zebrafish was carried out at 56 hpf in methylcellulose by first making a fine lesion with a dissection pin, and then carefully removing the contents of the yolk sac by suction through a glass pipette. In both *Xenopus* and zebrafish care was taken to avoid damaging the blood vessels and maintain blood circulation.

Immunostaining was performed on 12 μm sections using rabbit anti-active Caspase 3 (1:500; BD Biosciences; 59565), rabbit anti-phospho-Ribosomal protein S6 (Ser235; 1:500; Upstate; 07-433), mouse anti-Islet1 (1:100; Developmental Studies Hybridoma Bank; 39.3f7), Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 594 goat anti-rabbit (both 1:1000; Invitrogen). Antigen retrieval was performed by steaming with 0.01 M sodium citrate (pH 6) prior to staining for pS6. Nuclei were labelled with 0.1 μg/ml DAPI (Invitrogen). Immunofluorescent sections were imaged with an Olympus Fluoview FV1000 laser-scanning confocal microscope or with a Nikon.

**Fig. 7. Activation of mTOR signalling is sufficient to maintain the progenitor pool.** (A,B) Activation of mTOR with bpV rescues pS6 to a level 70% of that of controls in ND retinas (top row), specifically in CMZ progenitor cells (bottom row). Dashed lines encircle the CMZ. n=4, 16 embryos/condition. (C-F) bpV treatment rescued the downregulation of genes observed following ND, without affecting the expression of stem cell markers. n=4, 16 embryos/condition. (B-E) Error bars show 95% confidence intervals; ***P≤0.001, *P≤0.05 (unpaired t-test). Scale bars: 100 μm (A, top panel); 50 μm (A, bottom panel); 50 μm (F).
BrdU/EdU labelling
To mark cycling cells, 5 mM EdU was bath applied to Xenopus embryos, or embryos were injected with BrdU or EdU 2 hours prior to fixation. For pulse chase experiments, 5 mM EdU was bath applied with 1% DMSO for 20 minutes before being washed out. BrdU incorporation was detected as previously described (Agathocleous et al., 2009), and EdU detection was by Click-iT chemistry performed in accordance with the manufacturer’s instructions (Invitrogen). Flow cytometry was performed using a CyAN flow cytometer (Beckman Coulter) and analysed using FlowJo software (Tree Star). Statistical analysis was determined by Student’s t-test or one-way ANOVA.

In situ hybridisation
In situ hybridisation was performed on 20 μm sections as described previously (Xue and Harris, 2012) using digoxigenin-labelled antisense mRNA probes against rx1 (Mathers et al., 1997), atoh7 (Kanekar et al., 1997), asc11, neogenin2 (a gift from Dr Anna Philpott, University of Cambridge, UK), ath3 (Takebayashi et al., 1997), c-Myc and n-Myc (Bellmeyer et al., 2003), sax2 (Mizuseki et al., 1998), p27Xic1, cyclin A2 ( Howe et al., 1995), cyclin D1 (a gift from Dr Tim Hunt, CRUK, UK) and hes1 and hes4 (a gift from Dr Muriel Perron, Université Paris-Sud, France). Images were acquired using a Zeiss Axiostar microscope and Q-imaging camera and staining intensity was analysed using ImageJ. Intensity measurements were normalised to background intensity, then the intensity ratio of experimental conditions relative to controls was determined. Statistical significance was tested using a Student’s t-test.

Explant cultures
Stage 41 Xenopus embryos were deprived of their yolk for 24 hours prior to retinal dissection. For ex vivo re-feeding conditions, explanted retinas from control and nutrient-deprived embryos were cultured in a 60% solution of Leibovitz L-15 medium (Sigma) containing galactose and amino acids as nutrient sources. For ex vivo ND, retinas were cultured in 1× MBS, a salt buffer without nutrients. In proliferation experiments, explants were cultured for 22 hours at 20°C then incubated with EdU for 2 hours prior to fixation.