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When cell cycle meets development

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Summary

The recent Company of Biologists workshop 'Growth, Division and Differentiation: Understanding Developmental Control', which was held in September 2011 at Wiston House, West Sussex, UK, brought together researchers aiming to understand cell proliferation and differentiation in various metazoans, ranging from flies to mice. Here, we review the common themes that emerged from the meeting, highlighting novel insights into the interplay between regulators of cell proliferation and differentiation during development.

Key words: Cell cycle, Differentiation, Neurogenesis, Cyclin-dependent kinase (Cdk)

Introduction

Over the last few decades, there have been huge advances in our understanding of cell cycle regulation, but how core cell cycle regulators are linked to the onset of differentiation and how developmental cues control the cell cycle and tissue homeostasis in different developmental situations remain to be determined. The recent Company of Biologists workshop 'Growth, Division and Differentiation: Understanding Developmental Control' brought together researchers at different stages of their careers, from graduate students to experienced researchers, who focus on the connections between proliferation and differentiation. The purpose of the meeting, which was expertly organised by Nancy Papalopulu (University of Manchester, UK) and Anna Philpott (University of Cambridge, UK), was to promote discussions and expedite further research into this important area, through talks and evening discussion sessions.

Among the most important questions to be addressed in developmental biology are: (1) cell division and differentiation cannot happen at the same time, so how are these processes coordinated and how do cells arrest before they differentiate; (2) how is cell multiplication coordinated in different cell lineages in a specific organ and between different organs; (3) how is cell movement/migration coordinated with cell division; (4) how is cell division coordinated with apoptosis; and (5) how is the fate of multiplied cells determined in different cell lineages and organs?

Cell cycle progression is promoted by the activity of cyclin-dependent kinases (Cdks) (Morgan, 2007). Low levels of Cdk activity are sufficient for cells to transit from G0/G1 into S phase, whereas high levels of Cdk activity are required for cells to go through mitosis. For the G0/G1-S phase transition, Cdk4 and Cdk6 pair with D-type cyclins, whereas Cdk2 pairs with cyclin E and A. Individually, none of these G1-S Cdks or cyclins is essential in the mouse, but the loss of two or more usually results in embryonic lethality. During mitosis, Cdk1 pairs with cyclins B and A, all of which are essential for development. Cells can be arrested by the expression of Cdk inhibitors (CKIs) such as $p27^{Kip1}$ (Cdkn1b) and $p21^{Cip1/Waf1}$ (Cdkn1a), and, during S phase, regulators such as Geminin (a DNA replication inhibitor), the $CRL4^{Cdt2}$ (Cul4-associated factor 2) ubiquitin ligase complex and PCNA (proliferating cell nuclear antigen) control proper DNA replication. One of the important substrates and downstream effectors of the Cdk pathway is the retinoblastoma protein (Rb). Rb is a transcriptional repressor of E2F transcription factors and not only plays a crucial role in the G1-S transition but also in differentiation. The detailed functions of many cell cycle regulators have been described in vitro but in many cases their in vivo functions are not well understood (reviewed by Gopinathan et al., 2011).

The key regulators of differentiation during development are, to some extent, cell type-specific, but there are a number of common pathways, such as the Notch, Wnt, fibroblast growth factor (FGF) and Sonic hedgehog pathways, as well as common transcription factors, such as Sox family members, Oct4 (Pou5f1) and Myc. Different combinations of these signalling pathways and transcription factors are employed depending on the cell lineage or tissue. For example, in muscle development, the basic helix-loop-helix (bHLH) transcription factors MyoD and Mef2 (myocyte enhancer factor 2) play an important role, whereas in neurodevelopment transcriptional regulators such as Hes (hairy and enhancer of split), Neurogenin, and the bHLH transcription factors Math (atonal homolog) and Mash1 (achaete-scute complex homolog) are determining factors (reviewed by Gotz and Huttner, 2005; Guillemot, 2007).

The topics covered in this workshop included the regulation of differentiation by cell cycle regulators and by signalling pathways, and new technological advances in the field. This workshop was a forum for intense discussion and debate; in the evenings, the discussions continued around many of these specific themes. In the first evening session, each scientist was asked to introduce themselves and their research to facilitate discussions and interactions. The next evening, students and postdoctoral fellows were paired up to present their ideas for a collaborative grant proposal. On the last evening, passionate discussions focused on the 'next big question' in this field of research. These informal discussions contributed substantially to the friendly tone and success of the workshop – although it cannot be excluded that ghosts (spirits) of Wiston House guided through the workshop and influenced the discussions without any of the attendees noticing.

Below, using the key themes and topics as a template, we review the research and ideas that were presented at the meeting.

Regulation of differentiation by core cell cycle regulators

Neurodevelopment

The development of the neural system relies on the controlled expansion of certain cell lineages, and this was one of the focus points at the workshop. Despite many years of investigation, it is still unclear how the tight coupling of cell division and proliferation is

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achieved on a molecular level. Federico Calegari (German Research Foundation Research Centre and Cluster of Excellence for Regenerative Therapies, Dresden, Germany) presented elegant work, in which he manipulated the length of the G1 phase of the cell cycle by overexpressing Cdk4/cyclin D complexes in the adult mouse hippocampus. This favours the expansion of neural stem and progenitor cells and inhibits neurogenesis (Artegiani et al., 2011). The observed effect could be reversed by decreasing Cdk4/cyclin D expression. This work suggests that the length of G1, Cdk4/cyclin D levels and differentiation are intricately linked. Interestingly, though, Cdk2/cyclin E, a more potent driver of the cell cycle in other cell types, had no effect in this context. This difference could be caused by the role of cyclin D in transcriptional regulation in addition to cell cycle progression (Bienvenu et al., 2011).

A similar theme was continued by Philipp Kaldis (IMCB, Singapore), who analysed mice lacking both Cdk2 and Cdk4. Double-mutant knockout (DKO) mice die at ~E15 due to a heart defect, but there is also a defect in the brain associated with a loss of the intermediate zone and cortical plate. To identify the reasons for this phenotype, embryonic neural stem cells (NSCs) from DKO embryos were analysed. DKO NSCs displayed only minor proliferation defects (in contrast to mouse embryonic fibroblasts) but were prone to differentiation. This was partially due to an increase in G1 length but could also be caused by the hypophosphorylation of Cdk substrates. Such a Cdk substrate in *Xenopus* neural development, the proneural transcription factor Neurogenin 2 (Ngn2), was presented by Anna Philpott (University of Cambridge, UK). In proliferating cells with high Cdk activity Ngn2 is phosphorylated at many sites, rendering it unable to drive neuronal differentiation. The ability of Ngn2 to induce differentiation gradually increases depending on the number of Cdk sites that are dephosphorylated, and this quantitatively depends on the level of Cdk activity (Ali et al., 2011). Consequently, as G1 lengthens and Cdk activity decreases, Ngn2 becomes increasingly hypophosphorylated, resulting in the expression of the bHLH transcription factor NeuroD (neurogenic differentiation factor) leading to differentiation. Therefore, Ngn2 phosphorylation by Cdks represents a direct link between the cell cycle machinery and differentiation.

One of the many ways to lengthen the G1 phase is to increase expression of the Cdk inhibitor $p27^{Kip1}$. Therefore, studying the regulation of p27 is important. Renee Yew (University of Texas, San Antonio, USA) reported that *Xenopus* $p27^{Xic1}$, which shares homology with both mammalian p27 and p21, is degraded by ubiquitin-mediated proteolysis regulated by the ubiquitin ligase $CRL4^{Cdt2}$, but only when bound to PCNA and DNA (Kim et al., 2010). The degradation of p27 is complicated, though, as it can be dependent on the ubiquitin ligases Skp2 (S-phase kinase-associated protein 2), KPC (Kip1 ubiquitylation-promoting complex) and perhaps other ubiquitin ligases.

$CRL4^{Cdt2}$ is known best for its function in DNA replication, and this applies also to Geminin, which is an inhibitor of DNA replication but also has a role in neurogenesis in *Xenopus* (Kroll et al., 1998). Kristen Kroll (Washington University School of Medicine, St Louis, USA) presented data from mouse embryonic stem cells suggesting that, in this context, silencing Geminin has little effect on proliferation or self-renewal. However, Geminin was required for hyperacetylation of histones and thereby for promoting an open chromatin conformation at neural gene loci. This is surprising and suggests that cell cycle regulators might moonlight in contexts unrelated to proliferation. In support of this notion, data from Kristen Kroll's laboratory has shown that Geminin can interact with different chromatin remodelling complexes to regulate differentiation during *Xenopus* development (Lim et al., 2011; Seo et al., 2005).

Another example of a cell cycle regulator with functions outside cell proliferation was presented by Peter Sicinski (Dana-Farber Cancer Institute, Boston, USA) who analysed the functions of cyclin E in postmitotic neurons. His group has shown previously that cyclin E may display (Cdk) kinase-independent functions (Geng et al., 2007; Zhang, 2007). In most cases, Cdk2/cyclin E complexes act as positive regulators of proliferation by phosphorylating Rb, p27 and other substrates. Peter Sicinski's laboratory has now discovered that cyclin E is highly expressed in neurons that are not proliferating at all. Using proteomic approaches, cyclin E was shown to bind to Cdk5 in neurons and to inhibit Cdk5 activity by preventing binding of p35 (Cdk5r1) and p39 (Cdk5r2) (Odajima et al., 2011). Through this effect on Cdk5, cyclin E controls the formation of synapses and is involved in memory formation. This emphasizes that core cell cycle regulators can have functions distinct from their cell cycle roles at specific developmental stages.

Differentiation in other tissues

RB gene mutations lead to retinoblastoma in humans, but the mouse retina exhibits extra protection against retinoblastoma such that an additional Rb family member [p107 (Rbl1) or p130 (Rbl2)] needs to be deleted to induce tumourigenesis (Berman et al., 2009; Bremner et al., 2004; Chen et al., 2004). Rod Bremner (Toronto Western Research Institute, Canada) suggested that understanding p107/p130 function in the retina might provide strategies to block other Rb pathway tumours. He reported that, for tumour formation in the $Rb^{-/-}; p107^{-/-}$ mouse retina (Chen et al., 2004), increased E2F and Cdk2 activities are required. Moreover, there seem to be alterations to apicobasal polarity, suggesting that Rb has broader effects in the retina than in other tissues.

In addition to its tumour suppressor function, Rb has been widely implicated in differentiation. Jacqueline Lees (MIT, Cambridge, USA) investigated the differentiation of mesenchymal stem cells into osteoblasts and adipocytes (Calo et al., 2010). Loss of Rb favours the adipogenic fate over the osteogenic lineage, and this can also be seen in tumours arising from these cells. Mixed lineage tumours were only seen in *Rb*/*p53* (*Trp53*) double mutants. This indicates that Rb regulates the fate choice between bone and adipose tissue.

Regulation of the cell cycle and differentiation by signalling pathways

Another major theme of the meeting was the regulation of the cell cycle and differentiation by mitogenic or morphogenic signalling pathways. Ludger Hengst (University of Innsbruck, Austria) presented evidence for phosphorylation of mouse p27 (at tyrosine 88) by Jak2, which is activated by the cytokine interleukin 3 (Jakel et al., 2011). The phosphorylation of p27 at this residue results in a decrease in its ability to bind to, and to inhibit, Cdk2/cyclin A activity, and promotes p27 degradation leading to increased cell proliferation. This represents a link between mitogen signalling and the control of p27, which might be relevant not only for development but also for cancer.

Nancy Papalopulu (University of Manchester, UK) continued the theme of p27 regulation by signalling pathways, focusing on the *Xenopus* neural plate. The superficial cells in this tissue exhibit a polarized epithelial morphology and retain a progenitor cell fate, whereas the deep cells are not polarized and differentiate to primary neurons. Thus, this is a good model system in which to study the

influence of cell polarity on differentiation/proliferation. Although there seems to be a fundamental link between apicobasal polarisation and the proliferative properties of neural progenitors, from *Drosophila* to mammals, the mechanistic links between these two processes are not well defined. When an activated version of the apicobasal polarity regulator atypical protein kinase C (aPKC) is expressed, neurogenesis is blocked and proliferation enhanced (Sabherwal et al., 2009). Nancy Papalopulu reported that aPKC mediates these effects by binding to, and phosphorylating, a key component of the cell cycle. Thus, in the developing neural plate, the level of aPKC activity, through its direct effect on the cell cycle molecular machinery, can control neural differentiation. Helena Richardson (Peter MacCallum Cancer Centre, Melbourne, Australia) presented evidence for another mode by which aPKC regulates the cell cycle, in this case during *Drosophila* eye neural-epithelial development (Grzeschik et al., 2010). Activation of aPKC leads to dysregulation of the Hippo tissue growth control pathway, which is known to control cell proliferation (via *Cyclin E* transcription) and survival [via *Diap1* (*thread*) transcription]. During development, aPKC and its negative regulator Lgl [Lethal (2) giant larvae] may relay tissue tension/cell density cues to the core Hippo pathway components, thereby modulating organ growth. Whether similar controls of tissue growth by aPKC and the Hippo pathway occur during development of *Xenopus* or other vertebrates remains to be determined.

Further insight into the Hippo tissue growth control signalling pathway was provided by Nic Tapon (Cancer Research UK London Research Institute, UK), who described upstream regulation of the Hippo pathway in *Drosophila*, and reported the results of RNAi screening and mass spectrometry approaches that revealed new Hippo pathway components (Ribeiro et al., 2010). Ginés Morata (Centro de Biologia Molecular, Madrid, Spain) also described how the Hippo pathway is important for the competitive behaviour of clones in the *Drosophila* developing wing disc that are mutant for the polarity regulator *lgl* and that also express activated Ras (Menendez et al., 2010). However, during the genesis of these Ras-driven $lgl^{-/-}$ tumours, caspase activation and cell death are seen at the borders of the mutant and wild-type tissue, and the tumours can be eliminated unless they are able to develop a microenvironment that protects them from cell competition. The same effect was observed when mutants in the endocytosis regulator *Rab5* were combined with activated Ras. Remarkably, activation of the caspase Dronc (Nedd2-like caspase), which normally promotes cell death, was required for tumourigenesis owing to its effect on the secretion of the morphogens Decapentaplegic (TGFβ) and Wingless (Wnt), which drive cell proliferation in surrounding cells. These results show how tissue homeostatic mechanisms that control organ size during development can be usurped to promote the proliferation of mutant cells and drive tumourigenesis.

Continuing with the signalling theme, Muriel Perron (University Paris-South, France) described antagonistic interactions between Wnt and Hedgehog (Hh) signalling that regulate cell proliferation, quiescence and differentiation of stem/progenitor cells in the *Xenopus* retina (see Fig. 1). Wnt and Hh are expressed at opposite poles of the postembryonic stem cell zone of the retina, and whereas Wnt signalling maintains cells in the cell cycle, Hh signalling mediates the opposite effect. In addition, cross-talk occurs between the Wnt and Hh pathways at the level of transcription of the Hh regulator *Gli3* and the Wnt regulator *Sfrp1*, probably resulting in opposite gradients of pathway activity throughout the stem/progenitor zone. How stem versus progenitor cells specifically respond to this antagonistic Wnt/Hh signalling now needs to be determined. Fabienne Pituello (Centre de Biologie du Developpement, Toulouse, France) demonstrated the importance of Hh signalling in the switch to differentiation in the chicken spinal cord. Through in situ hybridisation screening, cyclin D1 and *Cdc25B* transcripts were observed to be upregulated at the time of neural differentiation and were shown to be induced by Hh signalling (Benazeraf et al., 2006; Lobjois et al., 2004). Cyclin D1, but not cyclin D2, has indeed been demonstrated to induce neuronal differentiation in the embryonic spinal cord (Lukaszewicz and Anderson, 2011). Thus, again, links are emerging between modification of the cell cycle – in this example by expression of specific cell cycle regulators – and the switch to a differentiation programme.

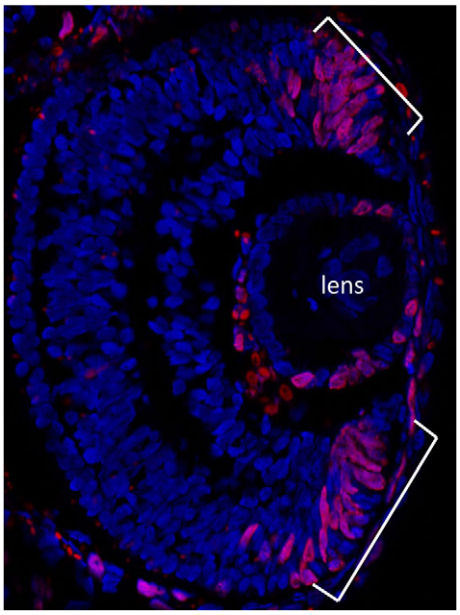

Fig. 1. The postembryonic stem/progenitor cell population in the periphery of the *Xenopus* postembryonic retina. Proliferating cells (labelled with EdU, red) are found within the periphery (brackets) of the retina. Nuclei are in blue, and the position of the lens is marked. Data from the Perron group (University Paris-South, France) suggest that the balance between proliferation and differentiation in this zone is finely tuned by antagonistic interactions between the Wnt and Hh signalling pathways.

Notch signalling is important for neural differentiation (Imayoshi et al., 2010). Ryoichiro Kageyama (Kyoto University, Japan) used sensitive reporters to show that Notch activity oscillates in the mouse embryonic neural tube (Shimojo et al., 2008). Relevant to this, Nick Monk (University of Sheffield, UK) demonstrated that this oscillation of Notch activity in neural differentiation could be modelled mathematically to fairly accurately represent the experimental observations (Momiji and Monk, 2009). Dr Kageyama also revealed that, in this system, the sustained upregulation of the Notch target Hes1 inhibits cell proliferation by leading to downregulation of cyclin D1 and cyclin E2. Interestingly, not only proliferation but also neural differentiation was inhibited by overexpression of Hes1 (Shimojo et al., 2008). Thus, in this situation, cell cycle exit does not correlate with differentiation.

Cell cycle control and differentiation – technical advances

Two talks stood out in highlighting new breakthroughs in technology that will improve our ability to dissect and understand cell cycle regulation and differentiation programmes. The first of

these was the plenary talk by Marc Kirschner (Harvard University, Cambridge, USA) on cell size control. How mammalian cells control their size is a fundamental question in cell biology. Using cutting-edge approaches, the dry mass or the protein mass of a cell can be measured accurately and, when compared with cell cycle parameters, has enabled for the first time a clear understanding of how cell size changes through the cell cycle in populations of growing cells in culture. Previous analysis had indicated that growth rate correlates with cell size throughout the cell cycle and that there is a rapid increase in growth rate in G1 followed by a constant exponential growth phase (Tzur et al., 2009). Using novel and more sensitive methodologies, these data were validated, suggesting that there is a sizing mechanism that operates in mammalian cells to reduce variation in growth rate and cell size between cells. This technology has far-reaching applications; for example, to enable the investigation of potential cell size regulatory genes that have been revealed by RNAi screens in *Drosophila* (Bettencourt-Dias et al., 2004; Boutros et al., 2004).

Bill Harris (University of Cambridge, UK) described novel imaging methods to monitor cell proliferation and cell fate in the zebrafish retina. In one such study, carried out in collaboration with Caren Norden's laboratory (Max-Planck Institute of Molecular Cell Biology and Genetics, Dresden), tools were generated to control and monitor cell cycle progression over time in live zebrafish. Using PCNA-GFP (which is nuclear in G1, forms punctuate dots in the nucleus when cells enter S phase, is diffuse in G2, and is then distributed throughout the cell in M phase) single cells could be monitored as they transited the cell cycle (see Fig. 2). Using this method, the length of time that individual cells spent in different phases of the cell cycle and how each phase of the cell cycle maps onto the apicobasal movements of nuclei during interkinetic nuclear migration could be monitored (Leung et al., 2011). This methodology will enable a greater understanding of proliferation and differentiation during vertebrate neural development by enabling an examination of how manipulations in signalling pathways or cell cycle regulators impinge upon cell fate decisions.

Conclusions

The research presented at this meeting, which came from studies of different organisms and developmental states, revealed several important insights into the connections between cell proliferation and differentiation: (1) cell cycle exit is not necessary to initiate differentiation; (2) instead, differentiation can be initiated by slowing of the cell cycle or by the expression of alternative cell cycle regulators; and (3) G1 length itself might be critical in determining whether a cell stays in a proliferative state or begins to implement cell fate decisions. Relevant to the last point, there is clear evidence that upregulation of several cell cycle inhibitors, including p27, Rb and Geminin (see Fig. 3), or downregulation of G1 Cdk/cyclins, which act to lengthen G1 phase and therefore cell cycle, can promote differentiation in different developmental settings. Although the majority of examples have focused on the G1-S phase transition, modulation of the G2-M phase regulator Cdc25B was observed to correlate with neural differentiation in the chicken spinal cord (Benazeraf et al., 2006). However, blocking the cell cycle via other means, for example via overexpression of Hes1 in the mouse neural tube, which results in inhibition of cyclin D1 and E2 expression, does not promote differentiation. It therefore seems that specific cell cycle regulators or a specific cell cycle state is required for the onset of differentiation. Nevertheless, future work will be needed to unravel the molecular mechanisms that couple proliferation and differentiation.

Morphogens and mitogens produced at specific developmental stages also profoundly influence the cell cycle and impact upon differentiation (see Fig. 3). These growth factor/morphogens (e.g. IL3-Jak2, Hh, Wnt and Notch) affect different cell cycle regulators depending on the developmental context. Indeed, depending on

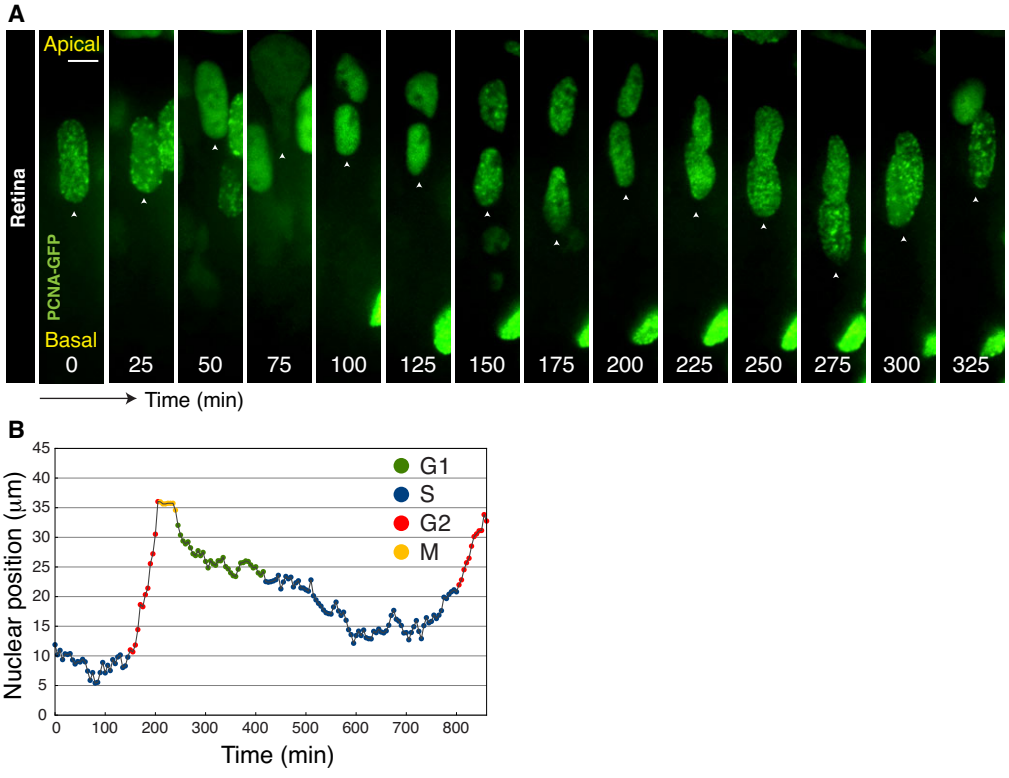

Fig. 2. Nuclear migration is tied to specific cell cycle phases. (**A**) Time-lapse imaging of zebrafish retinal/hindbrain nuclei expressing PCNA-GFP. Nuclei show stochastic movements in S and G1 with rapid directed apical motion in G2 phase followed by mitosis. Phases of the cell cycle can be identified by the differential distribution of PCNA-GFP (arrowheads) throughout the cell; PCNA-GFP is nuclear in G1, forms punctuate dots in the nucleus when cells enter S phase, is diffuse in G2, then is distributed throughout the cell in M phase. Scale bar: 10 μm. (**B**) The length of cell cycle phase versus apicobasal position for a single retinal nucleus. From Leung et al. (Leung et al., 2011).

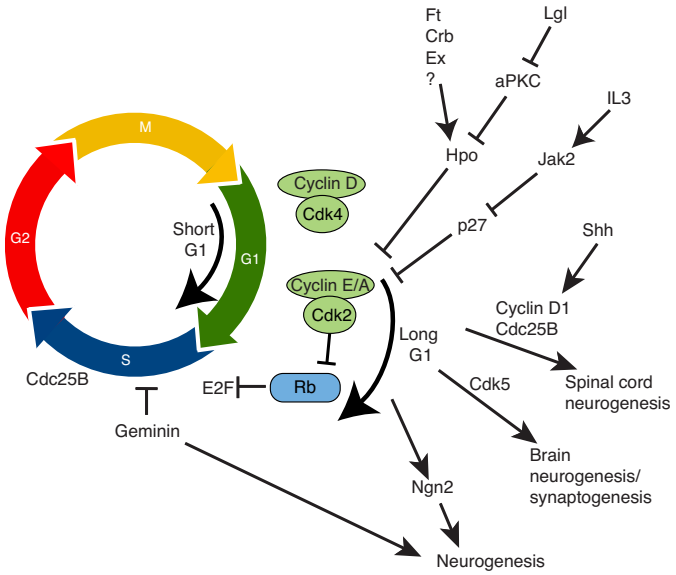

Fig. 3. A model of how signalling pathways affect cell cycle regulators and how cell cycle modulations affect differentiation. In *Drosophila* epithelial tissues, Lgl/aPKC and other upstream regulators [e.g. Fat (Ft), Crumbs (Crb), Expanded (Ex) and perhaps as yet unidentified factors] can modulate core Hippo (Hpo) pathway activity, which acts to inhibit *Cyclin E* transcription. In mammalian cells, IL3-Jak2 signalling inhibits p27 activity. In the absence of these positive signals, p27 inhibits G1-S phase progression, and the lengthening of G1 can promote differentiation. Rb and Geminin also inhibit progression into S phase. In the chicken spinal cord, sonic hedgehog (Shh) induces expression of cyclin D1 and Cdc25B, which correlate with induction of neurogenesis. In the mouse brain, cyclin E and Cdk5 can modulate neurogenesis and synaptogenesis. Neurogenin 2 (Ngn2) is also a key regulator of neurogenesis and is inhibited upon phosphorylation by Cyclin A/Cdk2 in *Xenopus*. Geminin can also promote neurogenesis in *Xenopus* and mouse cells by affecting the expression of neural differentiation genes. See text for further details.

context, morphogens can act either to promote proliferation or differentiation. Signalling pathways, such as Lgl/aPKC and the Hippo pathway, also act to limit cell proliferation to control organ size during development, possibly by responding to changes in tissue tension/cell density cues. In the development of tissues and organs, cells need to interpret signals from multiple external factors in order to undergo appropriate proliferation and differentiation. Disruption of the response to these external signals results in aberrant proliferation and blockage to differentiation that can lead to cancer.

From the research presented at this meeting, it is clear that the field has progressed in recent decades in its understanding of developmental control of proliferation and differentiation. The meeting prompted active discussion and, importantly, provided new scope for research collaborations to investigate fundamental questions in the relationship between proliferation and differentiation. However, the major message from the last night of after-dinner discussion at the meeting was that there are still many key questions that we have yet to fully comprehend, including how organism size is controlled at the cellular, organ and whole organism level, why some organisms can regenerate limbs/organs whereas others cannot, why there is so much heterogeneity in regulation during development, and what are the key events that are dysregulated to lead to hyperproliferation and the failure to differentiate in cancer? Clearly, future meetings are warranted to consolidate ideas and foster new approaches to interrogate these key questions in developmental control.

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Competing interests statement
The authors declare no competing financial interests.

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