Cell cycle trials in Salamanca

Workshop on G₂/M progression and associated checkpoints

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This EMBO workshop was organized by Angel Nebreda, Tim Hunt, Sergio Moreno and Paul Nurse and was held in Salamanca, Spain, September 6–9, 2001.

Polo-like kinases: wresting mitosis from the cyclin–CDK family

The plenary lecture was given by Stephen Elledge (Houston, TX), who introduced some of the themes that would recur during the meeting. He showed that, in budding yeast, the Bfa1/Byr4 protein, which makes up a two-component GAP (GTPase activating protein) with Bub2, is regulated by phosphorylation. The Bfa1–Bub2 pathway regulates exit from mitosis in response to the structure and position of the mitotic spindle and in response to DNA damage. Elledge presented evidence that Cdc5, the polo-like kinase in budding yeast, phosphorylates Bfa1 in anaphase to facilitate exit from mitosis. This phosphorylation was blocked by the spindle assembly and orientation checkpoints. In addition, DNA damage could block mitosis by two pathways: phosphorylation of the anaphase inhibitor securin to prevent its destruction; and hyperphosphorylation of Bfa1, which may help to prevent mitotic exit. During the meeting, it became apparent that the polo family had eclipsed the cyclin–CDK family as the most multi-faceted mitotic kinases (Figure 1; also see Donaldson *et al.*, 2001).

An effect of DNA damage in mitosis on the polo kinases was also demonstrated in animal cells. René Medema (Amsterdam, The Netherlands) showed that treating mitotic cells with agents that cause double-strand breaks arrested cells in mitosis and inhibited the polo-like kinase, Plk1, by a pathway that required the ATM kinase. Conly Rieder (Albany, NY) confirmed this effect (in his own, inimitable style) by irradiating different cell lines in mitosis with high-power laser pulses and showing that this caused them to delay in metaphase for several hours independently of the MAD-dependent spindle checkpoint (see below). These cells stained for phosphorylated histone H2AX, demonstrating that the DNA damage response pathway had been activated, and the delay could be abrogated with 2 mM caffeine, which inhibits the ATM kinase. Interestingly, when cells finally exited mitosis (in the absence of caffeine), they still stained for

Introduction

The time-trial stage of the annual 'La vuelta' cycle race was held in Salamanca in early September 2001. By coincidence, the cell cycle went on trial in Salamanca at the same time, at an EMBO workshop on G₂/M progression and associated checkpoints organized by Angel Nebreda and Sergio Moreno—and by Tim Hunt and Paul Nurse in their pre-Nobel-laureate stage! This was a highly enjoyable and stimulating meeting that covered a wide range of topics concerning the less fashionable part of the cell cycle: p53 was hardly mentioned and Rb got very short shrift. In this report, I will try to highlight some of the novel trends and concepts that emerged during the meeting rather than review all the talks. My apologies to those speakers whose talks I have been unable to include.

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Fig. 1. The many roles that Plk1 plays in the cell cycle. Plk1 has been implicated in the phosphorylation and activation (small arrows) or inactivation (blunt-ended lines) of the effectors of a variety of cell-cycle events. Plk1 may also be part of the negative feedback mechanism that prevents normal chromosome segregation when there is DNA damage.

phosphorylated H2AX, indicating that the damage may not have been repaired.

The importance of the polo kinases in initiating mitosis was highlighted by a number of speakers. Jan-Michael Peters (Vienna, Austria) showed that, in vertebrate cells, Plk1 was required for removing the bulk of the cohesin complexes from chromosome arms in prophase. This set the stage for the final separation of sister chromatids when the separase protease cleaved the last remaining cohesin subunits holding together the centromeres at the end of metaphase. Iain Hagan (Manchester, UK) showed that a mutation in a spindle-pole component called *stf1* or *cut12* by-passed the requirement for the Cdc25 phosphatase that is normally required to activate the cyclin-B/CDK kinase to initiate mitosis. This mutation caused the fission yeast polo kinase, *plp1*, to be recruited prematurely to the spindle. Furthermore, Hagan neatly demonstrated, using a temperature-sensitive mutant, that in these cells the *fin1* kinase was required for *plp1* to go to the spindle pole. Fumiko Toyoshima (Kyoto, Japan) showed that human Plk1 phosphorylated both cyclin B1 and its activator Cdc25C, causing them to move into the nucleus at mitosis by reducing their nuclear export. The physiological relevance of this to mitosis remained unclear. However, intriguingly, Marcel Dorée (Montpellier, France) showed that CDK1 bound to a non-phosphorylatable mutant of cyclin B1 could not be activated in the presence or absence of a nucleus. He presented data that indicated that cyclin B1 might need to be phosphorylated for the cyclin-B/CDK1 kinase to interact correctly with Cdc25C.

That the polo kinases were not exclusively concerned with regulating cyclin-CDK complexes at the beginning of mitosis

was demonstrated by Erich Nigg (Martinsried, Germany), who showed that Plk1 interacted with a novel centrosome component Nlp (ninein-like protein). When overexpressed in tissue culture cells, Nlp recruited the γ -tubulin ring complex (γ -TuRC) to the centrosome, and Plk1 appeared to cause Nlp to release γ -TuRC, leading Nigg to propose that this represented a step in centrosome maturation.

Dawn of the aurora era?

In addition to the prominent role played by the polo family of kinases, this meeting saw the aurora family of kinases begin to shine. Jason Swedlow (Dundee, UK) showed that aurora B was the major histone H3 kinase in mitotic cells and was regulated by interaction with the PP1 phosphatase. Histone H3 phosphorylation had been proposed directly to cause chromosome condensation, but Swedlow argued against this interpretation, favouring a role in making DNA more flexible, possibly to allow condensin complexes to act more efficiently. Kim Nasmyth (Vienna, Austria) illustrated another role for aurora (*lpl1*) in budding yeast. In a classic demonstration of solving problems through logic, he demonstrated that *lpl1* was required for chromosomes to attach properly to both poles of the spindle. He showed that *lpl1* formed part of an error-correction mechanism: when both sets of sister chromatids improperly attached to only one pole, *lpl1* was needed to detach microtubules from the kinetochores of one set of chromatids to allow these kinetochores to capture microtubules from the other pole. When chromosomes were properly attached to both poles, *lpl1* no longer localized to the kinetochores.

Chaperones and spindle checkpoints

Chaperone proteins made an unexpected appearance in a presentation given by Wolfgang Zachariae (Dresden, Germany). He showed that the CCT chaperonin complex, previously known for its role in actin and tubulin folding, had an important role to play in activating the anaphase promoting complex/cyclosome (APC/C). The APC/C acted as a multi-subunit ubiquitin ligase to control the degradation of a number of key mitotic regulators. This activity had been shown to depend on APC/C binding to a member of the WD40 family of proteins: in budding yeast, these were identified as Cdc20p and Cdh1/Hct1p. Zachariae found that the bulk of Cdc20 in a yeast cell was bound to the CCT chaperonin complex rather than to the APC/C. Furthermore, mutations in the CCT prevented Cdc20 (and Cdh1) from binding to the APC/C and, therefore, arrested cells in mitosis.

Cdc20 had first come to prominence as the target of the spindle assembly checkpoint. This checkpoint, mediated by the three MAD and three BUB genes plus the Mps1 kinase, had been shown to be essential for the proper segregation of chromosomes and, thus, for genomic stability. The spindle checkpoint was known to block APC/C-mediated proteolysis when activated, and substantial data indicated Cdc20 to be the target of the checkpoint. The prevailing view had been that the end-point of the checkpoint was the activation of MAD2 by unattached kinetochores and its consequent binding and inactivation of Cdc20p. However, two speakers challenged this view (also see Hoyt, 2001). Kevin Hardwick (Edinburgh, UK) found that there

are two important complexes involved: one with Mad3, Bub3, Mad2 and Cdc20; and a second with Mad1, Bub1 and Bub3. Bub1 and Bub3 appeared to be the most important proteins for genomic stability, perhaps because they have roles in addition to those at the spindle checkpoint. Simoetta Piatti (Milan, Italy) also found that the complex that bound Cdc20 consisted of several spindle checkpoint components. However, in contrast to Hardwick, she found that Mad1 and Bub1 were included in the complex with Bub3, Mad2, Mad3 and Cdc20. She also stressed the importance of Bub3, itself a WD40 protein, to the checkpoint and to genomic stability. Katja Wassmann (Paris, France) revealed that the interaction between MAD2 and the APC/C could also be negatively regulated by phosphorylation on MAD2.

There is more to separate than just cohesin

As mentioned above, a separate branch of the spindle checkpoint pathway in budding yeast had been shown to respond to the position of the spindle and was regulated through Bfa1–Bub2. This pathway regulated exit from mitosis, in particular the activation of the Cdc14 phosphatase. Angelika Amon (Boston, MA) presented evidence indicating some cross-talk between the two branches of the pathway. One of the primary effects of having satisfied the kinetochore branch of the spindle checkpoint and activating Cdc20 was the destruction of securin (Pds1). This activated separase (Esp1) to cleave the cohesins holding sister chromatids together. However, Amon found another role for separase: it was required to activate a small amount of Cdc14 by liberating Cdc14 from its partner Cfi1 in the nucleolus. This appeared to be required to initiate a timely exit from mitosis, because, in the absence of separase, cells were delayed in mitosis for up to 40 min. Frank Uhlmann (London, UK) had also found a role for separase in late mitosis. He showed that, in addition to cohesin, separase cleaved the Slk19 protein and that this was required to generate a stable spindle in anaphase. The physiological significance of this was further demonstrated by mutating the cleavage site in Slk19: the resultant, non-cleavable Slk19 partially destabilized spindles and, at higher levels, caused an increase in the rate of chromosome loss.

Microtubule dynamics: see how they Ran

The mechanisms that established the spindle were discussed by a number of speakers. Tony Hyman (Dresden, Germany) elegantly showed that adding only two proteins, XMAP215 (which stabilized microtubules) and XKCM1 (which increased the catastrophe rate, i.e. the rate at which microtubules switch from growing to shrinking), to purified tubulin was sufficient to recapitulate *in vitro* the dynamics of microtubules *in vivo*. Rafael Carazo-Salas (Heidelberg, Germany) showed that the Ran small nuclear GTPase, previously best known for its importance in nuclear transport, may play an equally important role after the nuclear envelope breaks down (reviewed in Dasso, 2001). He showed that, in its GTP-bound form, Ran caused microtubules originating at the centrosomes to grow longer, whereas, in its GDP-bound state, Ran blocked the interaction between centrosomes and chromatin. Allied with reasons to believe that Ran might be preferentially in its GTP-bound state on chromosomes, this led him to propose a model for how chromosomes might be

Fig. 2. Model for the signalling pathways that act at different stages of *Xenopus* meiotic maturation in response to progesterone. RINGO–CDK1 complexes stimulate the transition between G₂ and meiosis (M) I, whereas cyclin–CDK1 complexes are required for the transition between M1 and MII under the influence of Cdc25 and Myt1. The MAP kinase pathway arrests cells in MII in response to activation by mos, possibly through stimulating Bub1. Fertilization is required to release the cells from this arrest.

involved in setting up a bipolar spindle. In essence, microtubules that grow from the centrosomes would be stabilized and captured by the chromosomes due to the high concentration of Ran GTP around the chromatin. Mary Dasso (Bethesda, MD) also presented evidence that Ran could mediate spontaneous microtubule assembly, and she showed that the Ran GAP protein (which activates the GTPase activity of Ran) had to be covalently modified with SUMO-1 (a protein related to ubiquitin) to be targeted to the mitotic spindle.

The MAP kinase pathway in meiosis: who needs it?

In mouse meiosis, the task of stabilizing the spindle appeared to fall to a substrate of the MAP kinase pathway. Marie-Helene Verlhac (Paris, France) identified the MISS (MAP kinase interacting and spindle stabilizing) protein as a potential MAPK kinase substrate in a yeast two-hybrid screen and showed that, in its absence, the spindle failed to remain stable during metaphase II arrest: instead, the oocyte contained numerous microtubule asters. The role of the MAP kinase pathway in meiotic maturation provided the topic for one of several lively debates in the meeting (Figure 2; Abrieu *et al.*, 2001). The published literature had shown a requirement for the mos–MAPKK–MAPK pathway in meiotic maturation, notably in the frog oocyte. However, the general consensus from the talks at the meeting pointed more robustly toward a role in blocking cells in metaphase of meiosis II. Catherine Jessus (Paris, France) showed that inhib-

iting the MAPK kinase pathway with the MAPKK inhibitor, U0126, or with anti-sense morpholino-oligonucleotides directed against *mos*, delayed but did not block frog oocyte maturation. The terminal phenotype was an inability to maintain arrest in meiosis II, so oocytes tended to undergo parthenogenetic activation. This resembled the phenotype of both *mos*^{-/-} knock-out mice and *mos*-ablated starfish oocytes. Angel Nebreda (Heidelberg, Germany) introduced the RINGO proteins. These proteins were newly synthesized during meiotic maturation and were able to activate cyclin-dependent kinases 1 and 2 in a manner that by-passed all previously known regulatory pathways. RINGO-CDK complexes did not need to be phosphorylated to be activated, nor did they require the services of Cdc25. Moreover, exogenous RINGO was able to activate meiosis independently of MAP kinase and in the absence of protein synthesis. Takeo Kishimoto (Tokyo, Japan) clearly showed that the PKB/Akt protein kinase, and not MAP kinase or (remarkably!) the polo kinase, was instrumental in reinitiating meiosis in starfish oocytes. PKB/Akt did this by phosphorylating and inactivating the Myt1 kinase that kept cyclin-B/CDK1 in check.

Interestingly, PKB/Akt phosphorylated the same consensus site as recognized by the Rsk protein kinase, which is downstream of MAP kinase. James Maller (Boulder, CO) showed that Rsk could be the effector of meiosis II arrest because it was active in frog meiosis II and because exogenous Rsk was able to arrest cleaving frog blastomeres in mitosis in the absence of MAP kinase activity. Furthermore, he presented data that might connect arrest in metaphase of meiosis II with the spindle checkpoint. He found that Rsk could phosphorylate and activate the Bub1 kinase *in vitro* and that Bub1 was phosphorylated in a MAPKK-dependent manner during meiotic maturation. Until this meeting, there had been little evidence to link arrest in mitotic metaphase by the spindle checkpoint with the arrest of oocytes in metaphase of meiosis II. Helfried Hochegger (London, UK) contributed two further players to meiosis II arrest in frogs: cyclins B1 and B4. These B-type cyclins were newly synthesized during maturation and were required for the oocyte to progress from meiosis I to meiosis II. In their absence (as a result of adding anti-sense oligonucleotides), after meiosis I the meiotic spindle disassembled, MAP kinase was inactivated and the APC/C was partially dephosphorylated.

G₂ checkpoints: clasping the BRCT domain

Lastly, no meeting on G₂/M could be complete without a discussion of G₂ checkpoints. Both damaged DNA and unreplicated DNA had been shown to prevent cells from entering mitosis via a signal transduction cascade that involved the ATM or the ATR kinase (reviewed in Zhou and Elledge, 2000). The effector molecules that acted on the cell-cycle machinery had been identified as the Chk1 and Chk2/Cds1 kinases. In addition to the identification of the polo kinases as targets of these checkpoints, a second theme that emerged from the checkpoint talks at this meeting was that both Chk1 and Chk2 needed co-factors to block the cell cycle, and these co-factors had BRCT domains that had first been identified in the breast-cancer-related genes, BRCA1 and 2. Bill Dunphy (Pasadena, CA) had isolated the BRCT-containing protein, claspin, as a protein required for Chk1 to arrest the cell cycle in response to DNA replication blocks in

Xenopus extracts. This response depended on the ATR kinase that phosphorylated SQ motifs, and mutating four SQ motifs in Chk1 prevented its phosphorylation and activation by ATR. Similarly, Chk1 could not be phosphorylated and activated when claspin was immunodepleted, and claspin bound more strongly to the mutated form of Chk1. Thus, it appeared that claspin-binding was required for the subsequent phosphorylation and activation of Chk1. Paul Russell (La Jolla, CA) described the Mrc1 protein (mediator of replication checkpoint) that has weak similarity to claspin. He identified this protein in fission yeast as a component required for Cds1 to prevent mitosis when cells arrest in S phase. He showed that Mrc1 was only expressed during the S and early G₂ stages of the cell cycle and that exogenous expression in late G₂ phase caused inappropriate activation of Cds1. Tony Carr (Sussex, UK) presented data on the fission yeast BRCA1 homologue, Crb2, which linked recombination with the cell-cycle machinery. He showed that cells with mutant alleles of a B-type cyclin (*cdc13*) or Crb2 were radiation sensitive because they entered mitosis with damaged DNA, although the delay to mitosis was equivalent to that in wild-type cells. Crb2 was itself phosphorylated by cyclin-B/*cdc2* in G₂ phase, and alleles of Crb2 that could not be phosphorylated by cyclin-B/*cdc2* were radiation sensitive. Radiation sensitivity depended on the fission yeast RecQ helicase homologue, Rqh1, indicating that cyclin-B/*cdc2* controls the recombination machinery in G₂ phase via Crb2. Ashok Venkitaraman (Cambridge, UK) presented evidence on mammalian BRCA2 that may be related to this. He showed that, in the absence of BRCA2, mammalian cells spontaneously accumulated double-strand breaks without being exposed to genotoxins. He speculated that these breaks could have been caused during replication. Lastly, Mitsuhiro Yanagida (Kyoto, Japan) revealed an unexpected link between chromosome condensation and DNA damage repair. He first presented the structure of the condensin complex, revealed by atomic force microscopy as having two globular heads linked by a hinge region. The hinge region appeared to be the part that interacted with DNA. Then he showed that a mutation in one of the condensin subunits, Cnd2, not only prevented DNA condensation but also caused a defect in DNA repair and prevented the activation of the unreplicated DNA checkpoint. Although these cells were delayed in entering mitosis, once they did try to divide, the damaged DNA prevented them from properly segregating their chromosomes.

Afterword

In all respects, this was an excellent meeting. The talks were of a uniformly high standard, the atmosphere was relaxed and friendly, the discussions were lively and intelligent and the city of Salamanca never seems to sleep, so the more insomniac delegates could continue those debates in its beautiful plazas until the morning. Excellent, but exhausting.

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