Instituto Juan March
de Estudios e Investigaciones

81 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Co-sponsored by
EMBO EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

Workshop on

Cellular Regulatory Mechanisms: Choices, Time and Space

Organized by

P. Nurse and S. Moreno

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Introduction

Sergio Moreno and Paul Nurse

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INTRODUCTION

S. Moreno and P. Nurse.

One hundred and sixty years ago Theodor Schleiden and Jacob Schwann proposed the cell theory. This fundamental theory in Biology has two main implications: that every living organism is made out of one or more cells and that cells only arise by the division of pre-existing cells. In the last decade, there has been a lot of progress in our understanding of how cells divide. Most cells must complete four tasks during the cell division cycle. They must grow, replicate their DNA, segregate their chromosomes into two identical sets, and divide. Cell division is controlled by cyclin dependent kinases (CDKs). These protein kinases are periodically activated by their association with cyclins and their activity trigger the different phases of the cell cycle at the right time and in the right sequence. CDK/cyclin activity are themselves regulated by CDK inhibitors, by protein phosphorylation and by protein degradation. The combination of these regulatory mechanisms generate a relatively well characterized biological clock that regulates cell division.

CDK/cyclin complexes induce the onset of downstream events such as DNA replication and mitosis. DNA replication occurs during a short period of the cell cycle called S-phase. At the onset of S-phase, replication origins are activated by CDK/cyclin complexes. Recently there has been a lot of progress in the identification of proteins that bind and regulate replication origin activity. CDK/cyclin complexes also induce the cellular changes required for cells to enter mitosis. In this workshop the molecular details of the initiation of DNA replication, spindle assembly, chromosome movement and sister chromatid separation were discussed.

In multicellular organisms cell division depends on extracellular signals that ensures that a cell divides only when it is required. These signals activate or inhibit the CDK/cyclin complexes leading to cell proliferation or to cell cycle arrest followed by cell differentiation. Some of the cascades induced by these positive and negative signals

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and their integration with other developmental choices such as cell differentiation and cell death were an important issue of discussion.

In summary, several interesting problems in cell biology were the subject of this workshop. Questions such as: how do biological clocks work? How do cells co-ordinate cell growth with the cell cycle? How do signal transduction pathways stop cell division and induce cell differentiation? How is cell death regulated? How is the spatial organization of the cell recognised during cell division?

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Session 1: Cell cycle regulation

Chairman: Patrick H. O'Farrell

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THE MEIOTIC CELL CYCLE IN FISSION YEAST

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Imperial Cancer Research Fund

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The meiotic cell cycle can be considered as a modified mitotic cell cycle in which the S-phase between meiosis I and meiosis II is suppressed, chromosome segregation at meiosis I is reductional rather than equational, and recombination levels are increased. We have been investigating these processes to determine why they differ in meiosis compared to mitosis.

A gene *rec8* encoding a cohesion like molecule has been previously identified by the group of Jurg Kohli. We show that rec8p becomes associated with chromatin during meiotic S-phase and dissociates from chromatin and degraded during anaphase I. Cells lacking *rec8* exhibit an equational division at meiosis I instead of a reductional one. This meiotic cohesion is also required to promote recombination in the centromere region. We propose that rec8p cohesion is required for proper pairing of homologous chromosomes and for kinetochore orientation during meiosis I. Using a mutant which can initiate meiosis from G2 we show that rec8p is not present in these cells and that the division is equational. This suggests that rec8p is recruited to the chromosomes during meiotic S-phase and establishes pairing and kinetochore orientation, both processes being important to distinguish meiosis from mitosis.

Another protein important for promoting high levels of recombination during meiosis is the telomere binding protein taz1p. Taz1p is required for the stable association between telomeres and spindle pole bodies suggesting that this association is required for proper chromosome pairing and recombination.

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Positive and Negative Regulation of Initiation of DNA Replication in *S. pombe.*

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Cyclin-dependent kinases (CDKs) promote the initiation of DNA replication and prevent reinitiation before mitosis, presumably through phosphorylation of key substrates at origins of replication. In fission yeast, the $p65^{cdc18}$ protein is required to initiate DNA replication and interacts with the origin recognition complex (ORC) and the $p34^{cdc2}$ CDK. $p65^{cdc18}$ becomes highly phosphorylated as cells undergo the G1 --> S phase transition. This modification is dependent on $p34^{cdc2}$ protein kinase activity, as well as six consensus CDK phosphorylation sites within the $p65^{cdc18}$ polypeptide. Genetic interactions between *cdc18*$^+$ and the S-phase cyclin *cig2*$^+$ suggest that CDK-dependent phosphorylation antagonizes *cdc18*$^+$ function in vivo. Analysis of a mutant form of $p65^{cdc18}$ lacking CDK consensus sites indicates that phosphorylation directly targets $p65^{cdc18}$ for rapid degradation and inhibits its replication activity *in vivo*. Furthermore, the over-replication phenotype produced by expression of this mutant is resistant to increased mitotic cyclin/CDK activity, a known inhibitor of over-replication. Thus, $p65^{cdc18}$ is an example of a cellular initiation factor that is negatively regulated in vivo by CDK-dependent phosphorylation and proteolysis. Regulation of $p65^{cdc18}$ by CDK phosphorylation is likely to contribute to the CDK-driven "replication switch" that restricts initiation at eukaryotic origins to once per cell cycle (1, 2, 3, 4, 5).

Because defects in $p65^{cdc18}$ phosphorylation lead to a hyperstable and hyperactive form of $p65^{cdc18}$ which can promote high levels of over-replication *in vivo*, we wished to identify components of the Cdc18 proteolysis pathway in fission yeast. We have identified a new component of this pathway, encoded by the *sud1*$^+$ gene. *sud1*, like the previously identified *pop1*$^+$ gene, shares homology with the budding yeast *CDC4* gene. *sud1*$^+$ is not essential for viability, but is required to prevent spontaneous re-replication in fission yeast. Cells lacking *sud1*$^+$ accumulate high levels of $p65^{cdc18}$ and the CDK inhibitor Rum1, because they cannot degrade these two key cell cycle regulators. Through genetic analysis we have shown that hyper-accumulation of Rum1 contributes to re-replication in □ *sud1* cells, but is not the cause of the defect in $p65^{cdc18}$ proteolysis. Rather, Sud1 itself interacts with the ubiquitin pathway in fission yeast and binds to $p65^{cdc18}$ *in vivo*. Most importantly, Sud1- Cdc18 binding requires prior phosphorylation of the $p65^{cdc18}$ polypeptide at CDK consensus sites. These results provide a biochemical mechanism for the phosphorylation-dependent degradation of $p65^{cdc18}$ and other cell cycle regulators, including Rum1. Evolutionary conservation of Sud1/CDC4-related proteins suggests that phosphorylation-coupled proteolysis may be a general feature of nearly all eukaryotic cell cycles.

In addition to CDKs, members of the Cdc7 family of protein kinases are also required for the initiation of DNA replication in eukaryotes. We have purified the fission yeast Cdc7 homolog Hsk1 approximately 20,000-fold, to near homogeneity. Hsk1 has protein kinase activity and can autophosphorylate. Point mutations in several highly conserved regions of Hsk1

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inactivate the kinase *in vitro* and *in vivo*. Two of the mutant *hsk1* alleles examined block initiation of DNA replication and derange the mitotic checkpoint when overproduced, a phenotype consistent with a role for Hsk1 in the early stages of initiation. Hsk1 phosphorylates a six-member complex of minichromosome maintenance proteins purified from fission yeast specifically on the Cdc19 (Mcm2) subunit. These data are consistent with the hypothesis that that the essential positive function of Hsk1 at the G1/S transition is transduced via phosphorylation of Cdc19.

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REGULATING THE INITIATION OF DNA REPLICATION IN BUDDING YEAST

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All cells must co-ordinate the duplication of their genomes with cell growth and division. In eukaryotic cells, DNA replication initiates from multiple 'origins' throughout the S phase of the cell cycle. Our lab has been interested in characterising the initiation of DNA replication in the budding yeast *Saccharomyces cerevisiae*.

The first step in the assembly of initiation complexes involves the sequence specific binding of the six subunit Origin Recognition Complex (ORC) to an essential element within origins. This occurs immediately after initiation in the previous cell cycle and ORC remains bound throughout the cell cycle as well as during periods of quiescence. At the end of mitosis, an additional pre-replicative complex (pre-RC) is formed which requires the Cdc6 protein for both its formation and maintenance. A family of abundant proteins known as Mcm proteins enter the nucleus and bind to chromatin at this time in a reaction that also requires Cdc6p. Evidence will be presented indicating that Cdc6 acts as an ATP-dependent loader of the Mcm proteins.

The major cyclin dependent kinase, Cdc28p, together with the B cyclins (Clb's) play a dual role in regulating the initiation of DNA replication: they are essential for the firing of origins and they act to block the formation of pre-RCs. Thus, new pre-RCs cannot form until mitosis when Cdc28p- Clb kinase is destroyed and, therefore, re-replication within a single cell cycle is blocked.

Origin firing requires the action of a second protein kinase, Cdc7p. Like Cdc28p, Cdc7p protein levels do not vary during the cell cycle but Cdc7 kinase activity is periodic. This is, in part, because the regulatory subunit of the Cdc7p kinase, Dbf4, is an unstable protein whose levels fluctuate during the cell cycle.

Finally, evidence will be presented for a novel cell cycle dependency during S phase in which the firing of late origins is dependent upon completion of replication from earlier-firing origins. This dependency requires the Rad53 and Mec1 protein kinases and appears to act, at least in part, through the Dbf4 protein.

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COORDINATING CELL GROWTH WITH THE CELL CYCLE IN FISSION YEAST

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Eukaryotic cells co-ordinate cell growth with cell division at a point late in G1 called Start in yeast and the restriction point in animal cells. Beyond this point cells become committed to a new round of cell division and are unable to undergo cell differentiation. Genetic analysis in fission yeast has identified several cyclins that activate cdc2 in the cell cycle. cig1, cig2 and cdc13 are B-type cyclins while puc1 is more related to *S. cerevisiae* Cln3. In wild type cells cdc2/cig2 regulates entry into S-phase and cdc2/cdc13 entry into mitosis. cdc2/cig1 has been argued to have a minor contribution to the onset of S-phase, because cells deleted for $cig2^+$ and $cdc13^+$ can still perform S-phase but a triple deletion *cig1Δ cig2Δ cdc13Δ* blocks the cell cycle before the initiation of DNA replication. The role of cdc2/puc1 in the fission yeast mitotic cycle has not been clearly established.

The G1 phase of the cell cycle is characterised for being a period of low Cdk/cyclin activity. As cells exit mitosis two mechanisms, cyclin degradation and the synthesis of the Cdk inhibitor rum1, operate together to maintain the Cdk/cyclin complexes inactive during G1. We will present data showing that the length of the G1 phase (which is proportional to cell size in fission yeast) depends on a delicate balance between levels of negative (rum1 and srw1-APC) and positive (cyclins) regulators of cdc2. Early in G1, cyclin proteolysis and rum1 inhibition keep the Cdk/cyclin complexes inactive. As the cell grows during G1, cyclins begin to accumulate and CDK/cyclin activity eventually predominates. These Cdk/cyclin complexes phosphorylate the negative regulators (rum1 and srw1) promoting their degradation.

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Initiation of DNA replication at CpG islands in mammalian chromosomes

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CpG islands are G+C-rich regions about 1 kb long that are free of methylation and contain the promoters of approximately 50% of all mammalian genes. By contrast, bulk genomic DNA is comparatively G+C-poor and heavily methylated at CpGs. Given the well established relationship between gene transcription and DNA replication, we have tested whether CpG islands could be associated with replication origins in mammalian chromosomes. Predictions of this hypothesis are that CpG islands will be contained in nascent DNA strands smaller than their flanking regions and that they will replicate synchronously at the beginning of S-phase.

Analysis of *in vivo* replication intermediates at several hamster and human genes shows that the CpG island regions, but not their flanks, are present in very short nascent strands, suggesting that they contain or are adjacent to replication origins (ORIs). These results are further supported by the observation that CpG islands are enriched in a population of short nascent strands from human erythroleukaemic cells, suggesting that they constitute a significant fraction of endogenous ORIs. In agreement with these results, replication analysis of bulk CpG islands indicates that although dispersed along the chromosomes, they replicate synchronously and coordinately early in S-phase.

These results indicate that CpG island regions are used to initiate transcription and DNA replication and suggest a possible origin of the CpG islands as genomic footprints left on the chromosome by the replication initiation event.

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Modeling the control of DNA replication in fission yeast

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A central event in the eukaryotic cell cycle is the decision to commence DNA replication (S phase). Strict controls normally operate to prevent repeated rounds of DNA replication without intervening mitoses ("endoreplication"), or initiation of mitosis before DNA is fully replicated ("mitotic catastrophe"). The molecules and interactions that enforce these controls are beginning to be unraveled by elegant physiological, genetic and biochemical studies of fission yeast and budding yeast. We have distilled the evidence derived from fission yeast (*Schizosaccharomyces pombe*) into a molecular mechanism of "Start" control. Our model is based on the regulation of Cdc2 (a cyclin-dependent kinase) by synthesis and degradation of its cyclin partners, Cig2 and Cdc13, and by binding to a stochiometric inhibitor, Rum1. Using established principles of biochemical kinetics, we show that this model can describe not only wild-type cell cycles but also three, essentially different cell cycles observed in mutant strains of fission yeast:

- mitotic cycles of *wee1*$^-$ mutants, which are regulated by size control at Start, as a consequence of genetic defect in the mitotic inhibitor Wee1.
- endoreplication cycles, which are observed in the absence of mitotic cyclin Cdc13 or in cells overexpressing the inhibitor Rum1.
- rapid (or abbreviated) mitotic cell cycles observable in *wee1*$^-$ *rum1*Δ double mutants in the absence of any size control mechanism.

We identify the essential features of the mechanism that are responsible for the characteristic properties of Start control in fission yeast, and suggest crucial experimental tests of our model.

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Session 2: Cellular oscilators

Chairman: Martin Raff

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MITOSIS IN BUDDING YEAST

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The spindle checkpoint prevents the activation of the proteolytic machinery that triggers anaphase until the chromosomes have been correctly aligned on the mitotic spindle. We are analyzing this control genetically and biochemically.

Components of the checkpoint were identified by budding yeast mutants (*mad*, mitotic arrest deficient) and several of these proteins, including Mad2, are located to the kinetochores of vertebrate chromosomes that are not attached to the spindle. The Mad proteins also bind to Cdc20, an essential activator of the anaphase promoting complex (APC) that triggers exit from mitosis. Experiments in frog egg extracts demonstrate that cyclin proteolysis is regulated by the activity of the APC. We have shown that Cdc20 is the target of the spindle checkpoint by isolating Cdc20 mutants that bypass the checkpoint and showing that these mutants abolish the ability of the Mad proteins to bind to Cdc20.

Faithful chromosome segregation depends on the linkage between sister chromatids, which allows the sisters to orient towards opposite poles of the spindle. We follow chromosome segregation in living yeast cells by using a cassette that contains 256 copies of the Lac operator that bind a green fluorescent protein-Lac repressor fusion protein . We have used this system to investigate the dynamics of mitosis in wild type cells and mutants in the Cin8, Kip1 and Kip3 kinesins. Despite the strong genetic overlap between the functions of Cin8 and Kip1, the two motor mutants have distinct effects on anaphase, suggesting that different kinesins have different (although non-essential) roles in the function of the yeast spindle.

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Fission Yeast Pop1 and Pop2, Two Distinct Members of the CDC4 Family, Regulate Ubiquitin-Dependent Proteolysis of Substrates of the CDK

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We have identified the fission yeast mutant *pop1*, which becomes polyploid due to the abnormal accumulation of the CDK inhibitor Rum1. In the *pop1* mutant Rum1 becomes highly stabilised irrespective of cell cycle stage, which inhibits CDK activity, thereby resulting in re-replication. In addition to Rum1, we have found that the S phase regulator Cdc18 is also accumulated to high levels. The Pop1 protein contains F-box and WD repeats and is most homologous to budding yeast Cdc4. Biochemical analysis indicates that Pop1 is required for the ubiquitination and subsequent degradation of Rum1 and Cdc18 and binds Cdc18 in vivo.

We have recently cloned the *pop2+* gene encoding another WD repeat (and F-box) protein which shows a high homology to Pop1 and Cdc4. In the *pop2* disruptant, as in the pop1 mutant, Rum1 and Cdc18 become accumulated. Despite a similarity of both amino acid sequence and deletion phenotypes, Pop1 and Pop2 appear not to be functional homologues as overexpression of one does not rescue deletion of the other.

Both Rum1 and Cdc18 proteins contain consensus CDK phosphorylation sites and have been shown to serve as substrates for the CDK kinases. We have found that cell cycle-regulating proteins other than Rum1 and Cdc18 which contain CDK sites are also degraded in a Pop1- and Pop2-dependent manner. It, therefore, appears that Pop1 and Pop2 are required for a broader range of substrates than originally thought to be degraded through the ubiquitin pathway.

Kominami, K. and Toda, T. (1997) Fission yeast WD repeat protein Pop1 regulates genome ploidy through ubiquitin-proteasome mediated degradation of the CDK inhibitor Rum1 and the S-phase initiator Cdc18. Genes Dev. 11: 1548-1560.

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Molecular Genetics of Circadian rhythms in *Drosophila*

Michael Rosbash, Ravi Allada, Patrick Emery, Joan E. Rutila, Lea Sarov-Blat, Venus So, Vipin Suri. Howard Hughes Medical Institute, Department of Biology, Brandeis University, Waltham, MA 02254 and NSF, Center for Biological Timing.

Genetic and Molecular Analysis of the *Drosophila* circadian system has identified the *period* and *timeless* proteins (PER and TIM) as clock molecules that contribute to circadian pacemaker function.. Both genes show robust circadian rhythms of transcription, mRNA and protein expression. Furthermore, the two proteins interact to form a heterodimeric complex, and TIM levels respond to light, thereby tying the circadian pacemaker to photic stimuli.

To examine the role of TIM in the generation, maintenance and light sensitivity of the clock, we have made and analyzed transgenic flies that carry TIM under the control of a heat-shock inducible promoter. Behavioral and molecular analyses of the transgenic flies not only confirm TIM's status as a clock state variable but also indicate an important role in molecular regulation of the cycle. In a an arrhythmic *tim*^{0} background, a single heat shock recapitulates several features of the wild-type molecular cycle and provides important clues about the transcriptional as well as post-transcriptional circuitry involved in rhythm generation. Moreover, a heat shock even induces a burst of locomotor activity, suggesting that all of the machinery between TIM and activity is activated.

We are also continuing genetic efforts to find new genes involved in *Drosophila* rhythms. Our strategy is to search for suppressors or enhancers of either *per*L or *per*S, on the assumption that starting with a mutant rather than a wild-type clock genotype may reveal more components more easily. This genetic screen has identified a number of mutant candidates, which are in various stages of analysis.

We are currently characterizing a subset of mutants that apparently identify novel clock genes. Two mutants of particular interest are *cycle* and *Jrk*. Homozygous flies for both mutants are arrhythmic. *Jrk* is semi-dominant, whereas *cyc* is strictly recessive. Both genes are likely dosage-sensitive, as heterozygous deficiencies for the two regions show period-alterations. An additional clue that the mutants are interesting is that the homozygous arrhythmic flies have very low, non-cycling levels of PER and TIM protein. The two mutants also have low, non-cycling levels of *per* and *tim* mRNA as well. The defect is transcriptional, since mutant flies fail to manifest *per* and *tim* transcription as shown by nuclear run-on experiments. The transcriptional effects are much more severe than those associated with arrhythmic *per* or *tim* alleles, suggesting that CYC and JRK are upstream of *per* and *tim*. Very recent cloning efforts indicate that CYC and JRK are indeed transcription factors. Furthermore, the data suggest a strong link to the mammalian clock and a conserved, dedicated machinery for the circadian transcription of *per* and *tim*.

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GENETIC AND MOLECULAR ANALYSIS OF THE NEUROSPORA CIRCADIAN SYSTEM

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The circadian system in Neurospora is among the best understood of any organism and is the only such system described from the group of organisms including mammals that have a day-phase oscillator (Dunlap, Ann. Rev. Genetics 30, 579, 1996). There are three aspects to the timing system, Input, the clock itself or the Oscillator, and Output.

The oscillator is an autoregulatory feedback cycle, wherein the *frequency* (*frq*) gene encodes two forms of the FRQ protein, each of which can feed back to depress the level of transcript arising from the *frq* gene. An expectation and necessary consequence of such a negative feedback loop is that both *frq* RNA and protein cycle in abundance . This they do, each showing peaks in the subjective day with *frq* mRNA peaking about 4 hrs after subjective dawn (CT4) and FRQ peaking 4 hrs later at CT 8 (Aronson et al, Science 263, 1578, 1994; Garceau et al Cell 89, 469, 1997; Merrow et al, PNAS 94, 3877, 1997). This cycling is required for the clock to be sustained over many days. Similar regulation and phasing is seen for a putative component of the mouse circadian oscillator, *mPer1* (e.g. Tei et al, Nature 389, 512, 1997). The two forms of FRQ arise from alternative translation initiation sites; ambient temperature influences the clock by determining both the absolute amount of FRQ and the ratio between the two forms (Garceau et al Cell 89, 469, 1997; Liu et al, Cell 89,477, 1997). FRQ is phosphorylated as soon as it is made and is processively re-phosphorylated over the course of the day (Garceau et al Cell 89, 469, 1997), a modification that may play a role in regulating turnover. FRQ spends the early part of each day immediately following its synthesis in the nucleus, and the timing of its localization there appears to be regulated (Luo et al, EMBO J. 17, 1228, 1998). Nuclear localization is required for FRQ function. Within a few hours after FRQ acts to depress the level of its transcript, FRQ levels in the nucleus begin to fall although they continue to rise in the cytoplasm for a few hours before beginning to fall there also. Some FRQ persists in the cytoplasm till the mid-subjective night when positive acting factors, apparently encoded by the *wc-1* and *wc-2* genes, turn on synthesis of FRQ and begin the cycle again (Crosthwaite et al Science 276, 763, 1997). These *wc* genes are bona fide DNA binding transcription factors, the first such factors shown to be involved in a circadian clock.

Light resets the clock by rapidly inducing *frq* (Crosthwaite et al, Cell 81, 1003, 1995). This light induction requires the WC-1 protein, and is aided by the WC-2 protein (Crosthwaite et al Science 276, 763, 1997), both of which probably associate. Interestingly, the transcription of neither gene appears to be strongly rhythmic although mutations can lead to period length effects. These WC-1 and WC-2 proteins, required both for light-induction of *frq* and for sustained cycling in the dark, are clock proteins having sequences (PAS domains) similar to clock-associated proteins from the mouse (mPER, CLOCK) and the fly (PER), and also similar to light-response associated proteins from a number of systems, suggesting that clock molecules may have arisen from ancient proteins involved in light responsivity (Crosthwaite et al Science 276, 763, 1997). In a manner that appears to be almost identical in timing and kinetics to that described for Neurospora, the mouse circadian system is reset by light induction of mPer1 gene (Shigeyoshi et al, Cell 91, 1043, 1997).

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Clocks in nearly all non-homeothermic organisms interpret temperature steps as cues for dawn and steps down as cues for dusk.The absolute amount of FRQ protein is quite dependent on the ambient temperature so that even the low point in the daily FRQ cycle at 28°C is higher than the high point in the cycle at 20°C: this facts explains how temperature steps reset the clock. Following a step for 20°C to 28°C the amount of FRQ in the cell is always at or below the low point in the 28°C cycle, so the clock is reset to the time of day correponding to the low point in the cycle, approximately subjective dawn. Conversely, a high to low temperature step sets the clock to the time of day corresponding to the high point in FRQ levels, approximately dusk (Liu et al, submitted).

Output from the clock involves in part the clock regulated activation and deactivation of genes, so called "clock controlled genes or ccg's (Loros et al, Science 243, 385, 1989). 8 such clock-controlled (output) genes have been isolated through subtractive and differential hybridization, and they are involved in stress responses, development, and intermediary metabolism (Bell-Pedersen et al, PNAS 93, 13096, 1996). Circadianly regulated transcriptional activation of the clock-controlled gene *eas(ccg-2)* is mediated by an Activating Circadian Element in the promoter (Bell-Pedersen et al MCB 16, 513, 1996).

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Session 3: Growth control

Chairman: Andrew Murray

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REGULATION OF THE p16 TUMOR SUPPRESSOR

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The mechanism by which p16 arrests cell proliferation is relatively well understood. In contrast, very little is known about external stimuli and/or internal situations that trigger a p16-dependent cell-cycle arrest. Based on the fact that p16-deficient mice are viable and physiologically normal, we have hypothesized that p16 could act in response to abnormal situations such as cellular stresses or oncogenic activation. Previously, we found that primary cells respond to oncogenic Ras by upregulating both p16 and p53, and subsequently entering into a non-proliferative state reminiscent of senescence. This response can be viewed as an anti-tumorigenic response because murine p16-deficient cells become neoplastically transformed upon introduction of oncogenic Ras. We will present some recent data on the Ras-controlled pathways involved in this anti-proliferative response. In addition, we have analyzed the effect of an extensive series of chemotherapeutic drugs on both p16-containing and p16-deficient cells. We have found situations where p16 is effectively upregulated by specific chemotherapeutic drugs producing a p16-dependent cell-cycle arrest. Further analysis of these systems will hopefully provide clues about the cellular mechanisms that regulate p16.

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The ARF-p53 Pathway. Takehiko Kamijo[1,2], Frederique Zindy[2], Jason Weber[1,2], Mei-Ling Kuo[2], Martine F. Roussel[2], and Charles J. Sherr[1,2], Howard Hughes Medical Institute[1] and Department of Tumor Cell Biology[2], St. Jude Children's Research Hospital, Memphis Tennessee, 38105 USA.

The *INK4a/ARF* locus encodes two tumor suppressor proteins, $p16^{INK4a}$ and $p19^{ARF}$, that restrain cell growth by independently affecting the functions of the retinoblastoma protein (Rb) and p53. Disruption of this locus is one of the most common events in human cancer, perhaps second only to the loss of p53. *ARF/INK4a* contains two promoters and alternative first exons, designated 1α and 1β, whose RNA products are independently spliced to two common exons. Exon 1α, 2, and 3 encode $p16^{INK4a}$, an inhibitor of the cyclin D-dependent kinases that blocks their ability to phosphorylate Rb (1). In contrast, the exon 1β-2-3 transcript is translated in an alternative reading frame, with the resulting $p19^{ARF}$ product being composed of 64 amino acids derived from exon 1β and 105 residues encoded by the alternative reading frame of exon 2 (ref 2). The $p19^{ARF}$ protein can induce G1 and G2 phase arrest (2) in a manner that depends upon functional p53 (ref 3).

While neither $p16^{INK4a}$ nor $p19^{ARF}$ are detectably expressed during mouse embryogenesis, explantation of mouse embryo fibroblasts (MEFs) into culture induces the synthesis of both proteins, which accumulate progressively as the cells are continually passaged. In parallel, proliferation slows and gradually ceases after a finite number of population doublings. Rare cells that spontaneously emerge from crisis give rise to established cell lines (4). In about 75% of cases, establishment is associated with a loss of p53 function (5), whereas most of the remaining fraction sustains bi-allelic loss of the *INK4a-ARF* locus (3, 6). Although it was initially suggested that establishment of MEF lines occurred as a result of $p16^{INK4a}$ loss (7), deletion of *ARF* alone is sufficient to enable MEFs to grow continuously, even in the face of continued $p16^{INK4a}$ (as well as *INK4b,c,*and *d* gene) expression (3, 6). In MEF strains, then, ARF and p53 act epistatically in regulating the number of allotted population doublings that precede crisis. Interestingly, established MEF lines that lose p53 function rapidly become polyploid, whereas those that sustain bi-allelic *ARF* deletions tend to remain pseudodiploid, implying that p53 contributes independently to genetic instability. In agreement, tumors arising spontaneously in *ARF*-null mice can subsequently sustain p53 loss, indicating that $p19^{ARF}$ and p53 can collaborate in multi-step carcinogenesis (3).

Recently, two groups reported that $p19^{ARF}$ can bind directly to mdm2 to inhibit its function(s) (8, 9). The latter protein is encoded by a p53-responsive gene and acts in a feedback loop to inhibit p53-dependent transcription (10, 11) and to trigger p53 degradation (12, 13). It now seems likely that mdm2 acts as an E3 ubiquitin ligase that targets p53 for proteolysis (14). In addition, mdm2 regulates the nuclear export of p53, which is required to block p53-mediated transcriptional activation by accelerating p53 degradation (15).

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We have found that $p19^{ARF}$ can also interact directly with p53. $p19^{ARF}$ can form ternary complexes with p53 and mdm2, with all potential binary combinations being possible. Binding to p53 depends upon an exon 1β-coded N-terminal ARF domain (amino acids 1-62) that is necessary and sufficient to induce cell cycle arrest (16). In electrophoretic mobility shift assays using the recombinant proteins and labeled oligonucleotides containing consensus p53 binding sites, ARF physically interacts with p53-oligonucleotide complexes without affecting the stability of DNA binding. Not surprisingly, ARF depends upon functional p53 to transcriptionally induce mdm2 and $p21^{Cip1}$, and to arrest cell proliferation. In summary, ARF may potentially act in several ways: (i) as a p53 co-activator; (ii) as an antagonist of mdm2 ubiquitin ligase; and/or (iii) to prevent the mdm2-dependent nuclear export of p53.

Apart from its expression in explanted MEFs, the physiologic signals that induce *ARF* have so far remained elusive. MEFs and thymocytes derived from *ARF*-null animals exhibit unimpaired p53-dependent checkpoint responses after ionizing radiation damage, with MEFs undergoing cell cycle arrest and thymocytes exhibiting apoptosis. Therefore, *ARF* lies on a signaling pathway distinct from that activated by X-rays. Efforts to pinpoint signals that induce *ARF* will be discussed.

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FUNCTIONAL STUDIES OF THE RETINOBLASTOMA PROTEIN

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The biochemical activities of the retinoblastoma protein (pRB) and the signaling pathway in which it functions have been now been described in some detail based on work from a large number of labs over the past ten years. pRB acts as a negative regulator of transcription by binding to transcription factors such as E2F and holding them in an inactive state. During the mid-to-late stage of the G1 cell cycle phase, phosphorylation of pRB changes its conformation, the transcription factors are released from interaction with pRB, and key target genes are activated. The phosphorylation of pRB is directed by a group of cell cycle-regulated kinases, primarily from the cyclin D/CDK4 subfamily. In turn the cyclin D/CDK4 kinase activity is controlled by several mechanisms including the binding of p16, a CDK inhibitor.

In most human tumors, correct pRB function is lost by one of a series of mutations or epigenetic events. These mutations can hit RB, cyclin D, CDK4, or p16 genes, but seldom if ever are mutations found in more than one member of the pRB signaling pathway in the same tumor. This suggests that loss of pRB regulation of transcription is a critical step in tumorigenesis, and any method of losing pRB's negative regulation is sufficient to promote tumor development.

The next major issue that needs to be address by the field is to understand the functional role of the pRB signaling pathway. We have been examining the phenotype of mutations in the RBpathway in mice. One of the major targets of pRB, the transcription factor E2F-1, has been inactivated. Others have shown that E2F-1 can act as a oncogene in standard tissue culture experimental models. However, in the mouse loss of E2F-1 leads to a number of distinct phenotypes including tumor development, arguing the E2F-1 is also a tumor suppressor gene. This is the first example of a gene that can act both as an oncogene or tumor suppressor gene. Cells from these and other mice with mutations in the RB pathway are being used to examine the role of these proteins in differentiation, a setting where distinct roles for the various proteins can be discerned.

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PLANT Rb AND Rb-BINDING PROTEINS

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Plant cells respond to internal and external effectors which control their proliferation and growth. However, they have unique properties in terms of growth, cellular plasticity, body organization or development. Thus, it is conceivable that distinct molecular interactions, likely at the levels of regulation, substrate specificity or upstream effectors, may have evolved in plants. Over the past years, components of the plant cell cycle machinery, in particular, cdc2-like kinases as well as A- and B-type cyclins, have been identified and cloned, and its involvement in G2/M regulation has been studied.

Regulation of the G1/S transit in plants is much less understood. In mammalian cells, the product of the retinoblastoma susceptibility gene (Rb), a tumor suppressor protein, and other members of the family (p107 and p130), control the passage through the G1 phase and the G1/S transit. In human cells, Rb family members inhibit the activity of E2F transcription factors. A similar Rb regulatory pathway is not present in yeast cells. We have been interested in determining whether a Rb-pathway exists in plant cells and, if so, establish its similarities and differences with human cells. Among other aspects, these studies are relevant to understand:

(i) why tumor development is an extremely rare event in plants and plant cells are refractory to oncogenic transformation, and

(ii) the mechanisms by which a proliferation (and differentiation) program can be resumed from differentiated cells.

The presence of LXCXE Rb-binding motifs in both plant D-type cyclins (Soni et al., 1995, Plant Cell 7, 85) and in a plant DNA virus protein (Xie et al., 1995, EMBO J. 14, 4073) strongly suggested the existence of a Rb-like protein in plants. Later, we and others identified Rb-like proteins in maize (Grafi et al., 1996, PNAS 93, 8962; Xie et al., 1996, EMBO J. 15, 4900; Ach et al., 1997, MCB 17, 5077). Maize Rb-like protein has a conserved A/B pocket domain which mediates binding to LXCXE-containing proteins, including plant D-type cyclins, and it is expressed ubiquitously. Plant Rb-like protein can be phosphorylated in vitro by human CDKs and can interact with human E2F-1 (Huntley et al., 1998, Plant Mol. Biol., in press). Using plant Rb as a bait in a yeast two-hybrid screening, we have isolated several plant cDNAs encoding Rb-interacting proteins. Our results will be discussed in the context of whether plant cell cycle behaves as a "complex yeast cycle", as a "simple mammalian cycle" or, on the contrary, as a cycle with unique regulatory properties.

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Making choices through the TGF-b/SMAD Signaling Pathway

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The ability to respond to antiproliferative signals is critical for normal cell function and is often lost in cancer cells. In mammalian cells, studies on the mechanism that transduce and mediate antiproliferative responses have centered on TGF-b as a model system. TGF-b and the related activins and bone morphogenetic proteins (BMPs) can cause a lengthening or arrest of G1 phase, terminal differentiation, or programmed cell death. The presence and intensity of these responses depends on the cell type and the state of the cell. Focusing on different cell types that exhibit a G1 arrest response, we found that TGF-b inhibits the activity of cyclin-dependent protein kinases (Cdk) in all cases, but through a different set of rapid gene responses in each case. In Mv1Lu lung epithelial cells, TGF-b induces expression of the Cdk inhibitor p15Ink4b which cooperates with p27Kip1 levels already present in the cell to inhibit Cdk4/6 and Cdk2. In HaCaT human keratinocytes, TGF-b additionally induces the expression of p21Cip1 which complements the inhibitory action of p27Kip1 on Cdk2. In contrast, in MCF10A human mammary epithelial cells that lack *Ink4b*, TGF-b inhibits the expression of the Cdc25A tyrosine phosphatase with attendant accumulation of inhibitory tyrosine phosphorylation on Cdk4 and Cdk6. c-Myc expression is decreased by TGF-b in all cases. Thus, different cell types have evolved different mechanisms to respond (or not respond) to TGF-b with G1 arrest. The question then becomes, what is it that determines the specific set of gene responses elicited by TGF-b and related factors in each case.

We are addressing this question by first identifying the components of the TGF-b signal transduction pathways and then determining the basis of their specificity. Signal transduction by these factors involves three classes of molecules: a family of membrane receptor serine/threonine kinases, a family of cytoplasmic proteins -the Smad family- that serve as substrates for these receptors, and nuclear DNA-binding factors that associate with Smads forming transcriptional complexes. Signaling is initiated by binding of the growth factor to a specific pair of receptor kinases, an event that induces the phosphorylation and activation of one kinase, known as the "type I receptor", by the other kinase or "type II receptor". The activated type I receptor phosphorylates a subset of Smads, known as "receptor-regulated Smads" (R-Smads), which then move into the nucleus. On their way to the nucleus, R-Smads associate with the related protein Smad4, a tumor suppressor gene product. In the nucleus, this complex may associate with specific DNA-binding proteins that direct it to the regulatory region of target genes. The integrity of this signaling network is essential for normal development and tissue homeostasis, and its disruption by mutation underlies several human inherited disorders and cancer. The TGF-b and activin type I receptors, which have nearly identical kinase domains, interact with and phosphorylate Smad2 (or the closely related Smad3) which then interacts with DNA-binding factors such as The BMP receptors interact with Smad1 (or the closely related Smads 5, 8 or, in *Drosophila*, Mad) which do not recognize. Although the TGF-b and BMP pathways are well segregated from each other, their receptors and R-Smads are structurally very similar. The specificity of the receptor and Smad interactions in each pathway may therefore be dictated by discreet structural elements. We have identified structural elements that dictate the selective interactions between receptors and Smads, and between Smads and transcription factors. These determinants provide a basis for understanding the specificity of TGF-b signal transduction

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pathways. Cell-specific repertoires of molecules such as Fast1 that tether Smad complexes to specific gene regulatory regions may, in turn, specify the nature of the TGF-b response in different cell types.

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Session 4: Developmental choices: proliferation, differentiation and cell death

Chairman: Joan Massagué

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The dual nature of signalling pathways that regulate cell proliferation and cell survival. Gerard I. Evan, Imperial Cancer Research Fund Laboratories, 44, Lincoln's Inn Fields, London WC2A 3PX, UK.

Cancers arise through the accumulation of mutations that compromise control of cell proliferation, differentiation and programmed cell death (apoptosis). Deregulation of the c-*myc* proto-oncogene is an example of a mutation that disrupts cell growth control and makes cells independent of mitogens for cell cycle progression. However, activation of c-*myc* also triggers apoptosis in cells deprived of excess survival factors (Evan et al., 1992). The dual effect of c-*myc* appears to serve as an inbuilt restraint to the propagation of potential tumour cells harbouring activated c-*myc* (Harrington et al., 1994).

If c-*myc* oncogenesis is suppressed by apoptosis, then factors that suppress cell death should enhance the oncogenic potential of c-*myc*. We have shown that expression of the anti-apoptotic oncogene *bcl*-2 co-operates with c-*myc* by this mechanism (Fanidi et al., 1992). Another important suppressor of apoptosis is the action of "survival factors" - cytokines that trigger signalling pathways that suppress apoptosis. We have dissected one such pathway: that activated by the promiscuous cytokine IGF-I (Harrington et al., 1994). Upon ligation by IGF-I, the IGF-I receptor activates the intracellular signalling enzyme PI 3K via Ras. PI 3K in turn activates the serine/threonine kinase PKB/Akt, whose action alone is sufficient to replace exogenous IGF-I and suppress oncogene-induced cell death (Kauffmann-Zeh et al., 1997). Intriguingly, although Ras is a necessary intermediary in this "survival" signal, it also activates an opposing pro-apoptotic pathway via the Raf kinase. The fate of the cell appears to depend upon the balance between these two opposing signals: which one predominates depending upon cross talk with other signalling pathways obtaining in the cell. Importantly, activation of the Ras protein alone, as would occur following oncogenic mutation, triggers an auto-destruct death programme that removes the potential tumour cell. Like Myc, therefore, Ras activates an innate "booby trap" that censors cells that acquire potentially neoplastic mutations. We believe that such booby traps are major restraints to the ontogeny of cancers.

We have also investigated the molecular mechanism by which c-Myc triggers apoptosis. We have shown that c-Myc-induced apoptosis in fibroblasts requires an interaction on the cell surface between the CD95 (Fas/Apo-1) receptor and its ligand. It appears that c-Myc recruits an endogenous Fas death signal and in some way uses it to engage the basal apoptotic machinery of the cell (Hueber et al., 1997). This might suggest that dysfunctional Fas signalling would be a frequent occurrence in cancer. However, we have uncovered another booby trap: blockade of Fas signalling leads to a profound growth arrest in affected cells, effectively curtailing their further neoplastic progression (Zörnig et al., 1998).

A paradigm emerges whereby evolution has linked signal transduction pathways with neoplastic potential (growth and survival pathways) with growth inhibitory mechanisms that defeat the growth promoting pathway unless inhibited by an intersecting signal. Thus, cell propagation can only arise through the confluence of appropriate signalling pathways that gate each others' growth inhibitory activities.

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AN INTRINSIC TIMER AND EXTRINSIC SIGNALS CONTROL THE TIMING OLIGODENDROCYTE DIFFERENTIATION

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In most vertebrate cell lineages precursors cells divide a limited number of times before they stop and differentiate. In no case is it clear why the cells stop dividing when they do. The stopping mechanisms are important, as they influence the timing of differentiation and the number of differentiated cells generated. We have been studying the stopping mechanism in the oligodendrocyte lineage in the rodent optic nerve.

The oligodendrocytes develop from dividing precursor cells that migrate into the optic nerve early in development. The precursor cells are stimulated to divide mainly by PDGF, but even when they are cultured in saturating amounts of PDGF, the precursor cells isolated from a postnatal day 7 nerve divide no more than 8 times before they stop and differentiate(1). The timing of this cell-cycle arrest and differentiation depends on an intrinsic timer (1, 2), which consists of at least two components— a counting component that measures elapsed time and an effector component that stops the cell cycle and initiates differentiation when time is reached(1). I shall present evidence that the effector mechanism is regulated by thyroid hormone(1) and that the cyclin-dependent kinase inhibitor p27 is part of both the counting and effector mechanisms(3, 4, 5).

It seems very likely that similar mechanisms operate in many other cell lineages.

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A CONSERVED SIGNAL TRANSDUCTION CASCADE INVOLVING NITRIC OXIDE SYNTHASE MEDIATES BEHAVIORAL AND CELL CYCLE RESPONSES OF DROSOPHILA TO HYPOXIA.

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I will review some studies of the onset of G1 phase during Drosophila development, and then will focus on new studies of the response the to hypoxia.

In many species, early embryonic cell cycles lack gap phases. In Drosophila, a G2 phase first appears in cell cycle 14, and a G1 phase appears in cell cycle 17. The changes in cell cycle regulators that accompany introduction of a G1 provide some insights into what a G1 is and what step is necessary to escape this stable period of

Our results suggest strong parallels with the control of the cell cycle in yeast. Cyclin A and cyclin E activities inhibit protein destruction having features characteristic of APC mediated destruction. We suggest that mutual antagonism of APC and cyclin A plays a fundamental role in creating two stable stages of the cell cycle: a stage where APC is inactive and cyclin A is present - S phase through metaphase - and a stage when APC is active and cyclin A is absent - anaphase through G1. During development, a G1 appears when APC mediated destruction dominates. The stability of G1 can be understood in terms of the mutual antagonism of APC and cyclin A. While cyclin A can drive S phase, it is extremely unstable and is inhibited by Roughex (a Drosophila analog of Sic1/Rum1) during G1. Cyclin E is unaffected by APC mediated destruction or by the Roughex inhibitor, and its transcriptional induction leads to accumulation of active kinase inactivation of APC (and also destruction of Roughex). Hence, cyclin E is specialized in tripping the system of mutual inhibition from APC domination to cyclin A domination. Perhaps cyclin B and Fizzy (Cdc20 homolog) play the reciprocal role, switching it back to APC domination at the metaphase anaphase transition.

The response of cells to hypoxia plays an important role in tumor progression. We have examined the response to hypoxia in a powerful model system, Drosophila. Reduced oxygen tension induced a cell cycle arrest and provoked larvae to leave their food and wander (this "exploratory" behavior contrasts to the usual "sitting" behavior). Results suggest that hypoxia activates nitric oxide synthase, whose product, nitric oxide (NO), activates cyclic GMP synthase, whose product, cGMP, activates protein kinase G (PKG). This cascade is required for survival, behavioral responses and cell cycle responses. This NOS/PKG pathway signals vasodilation in response to hypoxia in humans and we suggest that this signal transduction system is part of a highly conserved response to hypoxia.

Hypoxia (and apparently the NOS/PKG pathway) activates cyclin/Cdk1, and inactivation of a mutant form of Cdk1 demonstrated that its activity is required to arrest S phase in response to hypoxia. Although Cdk1 normally promotes mitotic cell cycle events, its unusual activation during hypoxia provokes a pseudo-mitotic state characterized by histone phosphorylation and appears to arrest the cycle in mid-S phase.

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AVIAN *HAIRY* GENE EXPRESSION IDENTIFIES A MOLECULAR CLOCK LINKED TO VERTEBRATE SEGMENTATION AND SOMITOGENESIS

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In vertebrate embryos, the most obvious metameric structures are the somites. They constitute the basis of the segmental pattern of the body and give rise to the axial skeleton, the dermis of the back and all striated muscles of the adult body. In the chick embryo, a somite pair is laid down every 90 min in a rostro-caudal progression, and a total of 50 somite pairs are formed during embryogenesis. Several hypotheses have been put forward to account for the mechanism of somite generation. It was proposed that somitic precursor cells are exposed to a "clock" which coordinates or synchronises the behaviour of cells that are destined to segment together into a somite. At the molecular level, little is known about the machinery driving segmentation in vertebrates. Numerous vertebrate homologues of the *Drosophila* segmentation genes have been identified but are not expressed during somitogenesis. This, therefore, has supported the view that segmentation arose independently in vertebrates and invertebrates.

We have identified and characterised *c-hairy1*, an avian homologue of the *Drosophila* segmentation gene, *hairy*. In *Drosophila*, *hairy* is a member of the pair-rule genes which are the first to reveal the prospective metameric body-plan of the fly They are expressed in a series of stripes with alternate-segment periodicity, and are used in combination to establish the future segmental periodicity of the embryo. *c-hairy1* is strongly expressed in the presomitic mesoderm where its mRNA exhibits a cyclic posterior-to-anterior wave of expression whose periodicity corresponds to the formation time of one somite (90 min). This wave is not due to massive cell displacement along the antero-posterior axis, but arises from pulses of *c-hairy1* expression that are coordinated in time and space. Analysis of in vitro cultures of isolated presomitic mesoderm demonstrates that rhythmic *c-hairy1* mRNA production and degradation is an autonomous property of the paraxial mesoderm and does not result from caudal-to-rostral propagation of an activating signal. Blocking protein synthesis does not alter the propagation of *c-hairy1* expression, indicating that negative autoregulation of *c-hairy1* expression is unlikely to control its periodic expression. These results provide the first molecular evidence of a developmental clock linked to segmentation and somitogenesis of the paraxial mesoderm, and support the possibility that segmentation mechanisms used by invertebrates and vertebrates have been conserved.

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GENETIC CONTROL OF CELL PROLIFERATION IN MORPHOGENESIS

Antonio García-Bellido

The wing imaginal disc of Drosophila grows from an embryonic anlage of about 30 cells to an adult mesothorax of about 50.000 cells. Clonal analysis reveals that cell proliferation is intercalar and takes place throughout the entire anlage (1). The average cell division rate is of 8 1/2 hours. However individual cells pass through the cell cycle with variable speeds (with G1 periods of more than 16 hours and G2 periods of less than 30 min.). Ciclying times are not clonal but depend on cell interactions between neighbouring cells, by mutual recruitment, in synchronic clusters. Mitotic orientations in these clusters are planarly at random, but postmitotic cells allocate to fixed relative positions along the wing axes. At the end of the proliferative phase, in prepupal wings, cell proliferation follows a temporal regime related with prospective cell differentiation (veins, interveins, chaetae). The number of cells of the mature disc is constant, and invariant under experimental conditions of mitotic delay, acceleration or massive apoptosis (2,3,4)

What are the intercellular signals that trigger or stop mitosis, finally determining constant cell numbers (and organ shape)? Old and recent experiments have revealed a local control of cell proliferation, rather than cells responding to sources of morphogens. 1) Discs grow to final size irrespective of growing physiological conditions (5). 2) Dissociated cells are capable of reconstructing original patterns, with correct cell polarities, upon reaggregation (6). 3) Isolated cells are capable of proliferating to normal, albeit fractioned, patterns in "feeder layers" (7). 4) Genetic mosaics of mutations causing smaller or larger cells autonomously form normal patterns, integrated or not, with the surrounding territories, indicating that size computation is in number of cells not in physical distances (8). 4)

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Mosaics of mutations in genes encoding ligands, receptors, transducing signals and transcription factors reveal preferential allocations of daughter cells in a growing wing landscape, as defined by clonal restriction boundaries, of compartments and of veins (9). 5) New boundaries, in genetic mosaics cause proliferative "accommodation" of wild type cells to ful a primordium with all the intercalar positional values (10).

These findings have led to the proposition of a generative model, the Entelechia model, of morphogenesis (9). In this model individual cells exhibit surface labels that express scalar differences and planar polarity along two orthogonal axes, x and y. The amount of surface label depend on the level of Martial (M) gene products within each cell. The model assumes that the confrontation of cells on both sides of compartment borders causes an increase in their level of M gene expression. The resulting disparity between the M value of border cells and that of their neighbours induces the latter to divide. After each division the daughter cells increase their own M value, and allocate to the best matching value position among neighbours. The increase in M value at the borders therefore extends through the anlage in a cascade of proliferation. The Entelechia condition is reached when the border cells attain the species-specific maximal M values, and the value differences between adjacent cells become indistinguishable. Computer simulations reveal that this model accounts for the above experimental observations made on imaginal discs.

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Session 5: Spatial organization of the cell

Chairman: Paul Nurse

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ORIENTATION AND FORMATION OF AXES OF CELL POLARIZATION IN YEAST. John Chant, Molecular and Cellular Biology, Harvard University, Cambridge, USA.

The generation of asymmetry is one of the most fundamental problems of biology. Yeast cells dividing by budding provide a highly tractable system for deciphering the mechanisms by which cells polarize to become asymmetric. During vegetative growth by budding, yeast cells can polarize, bud, and divide in two spatial patterns: the haploid axial pattern in which newborn buds form at the junction of the previous mother and daughter cells, and diploid bipolar pattern in which newborn buds form at the poles of the ellipsoidal cells. Two important questions are the following. First, how does the cell choose sites for budding in the two defined patterns? Second, once a site for budding is chosen, how does the cell build an axis of polarization? These two questions have been separated genetically. One class of genes (*BUD1-BUD10*) is required for choosing bud sites in ordered patterns, but not for axis formation *per se*: mutants polarize and form buds in aberrant orientations. A second class of genes (*CDC24, CDC42, CDC43, BEM1*) is required for building the axis of cell polarization; mutants cannot polarize, and, as a consequence, they enlarge uniformly, rather than forming a bud.

The mechanism of axial budding

Bud3, Bud4 and Bud10 appear to be the landmark proteins that define the mother-bud neck in one cell cycle for axial budding in the next cell cycle. The central landmark protein appears to be Bud10, a single pass transmembrane protein which may function much like non-enzymatic receptors. Our work is focused to understand several issues concerning Bud10.

The temporal mechanism of axial budding. How is the action of Bud10 temporally controlled? Although Bud10 is present in the mother-bud neck for the entire cell cycle, *BUD10* mRNA is only present in G1. In order to examine the importance of *BUD10* periodic expression, we altered the cell-cycle periodicity of its expression to occur in the G2->M period. With altered periodicity of expression, Bud10 is unable to function and the protein is localized diffusely on the membrane. Remarkably, in the wild-type and the manipulated situations, Bud10 protein is detectable for the entire cell cycle. Therefore, it appears that we have defined a case where strict temporal control of gene expression is essential to product localization and function.

Cell type control of budding patterns. How is cell-type control of budding pattern mediated through Bud10? Recent work, in collaboration with Atsushi Fujita and colleagues, has suggested that the action of Bud10 is inhibited by a transmembrane molecule Rax1 in combination with a second protein, not yet well defined. The modulation of the action of a transmembrane signaling molecule, Bud10, by another membrane protein potentially represents a novel mode of signal regulation.

Bud10 signaling mechanism. Third, what is the mechanism by which Bud10 signals? Genetic evidence suggests that Bud10 acts through the Bud1-Cdc42 GTPase cascade. Through affinity and genetic methods, we are attempting to decipher the intervening proteins in this pathway.

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Control of cell polarization by Cdc42 GTPase.

How Cdc42, a Rho family GTPase, controls the polarization of the actin and microtubule cytoskeletons is a major unsolved problem of cell biology. Efforts are currently dedicated towards deciphering in molecular detail the pathway leading from Cdc42 to the cytoskeleton. Efforts have centered on the identification of Cdc42-binding targets. Recently, on the basis of the genomic sequence of yeast, we have identified two novel Cdc42 targets, Gic1 and Gic2. These partially redundant proteins clearly have an effect on cytoskeletal polarization, however the *gic1gic2* phenotype indicates that they cannot solely account for the effects of Cdc42 on the cytoskeleton.

In other recent work we have identified and characterized a yeast homolog of IQGAP proteins (denoted Iqg1 for IQGAP related), a class of proteins postulated to be important Cdc42 effectors in higher eukaryotic cells. However, analysis of Iqg1 function has led to the conclusion that this protein is a central regulator of actin ring formation and cytokinesis, rather than cell polarization.

Based on analysis of the Gic proteins and other candidate Cdc42 effectors (including PAK family kinases and possibly formins), there remains much to be learned about the mechanism of Cdc42 action. A likely possibility is that a key effector of Cdc42 has yet to be discovered; the opposite view is that all relevant effectors are known and that they simply work in combination. Currently, both possibilities are being investigated. Once relevant effectors have been clearly defined, we shall turn to understanding their mechanisms of action.

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THE SPATIAL ORGANIZATION OF MICROTUBULES BY PHOSPHORYLATION GRADIENTS: IMPLICATIONS IN CELL MORPHOGENESIS.

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We are trying to determine the number and nature of the general principles involved in the morphogenesis of eukaryotic cells. Presently, we envision several general principles: i) formation of high molecular weight complexes and supra-molecular structures through the stereo-specific recognition of proteins, ii) formation of dynamic supra-molecular structures through directed self assembly processes, iii) exploratory behavior of dynamic polymers, iv) self organization behavior of polymers, v) control of the spatial organization of these polymers by regulatory networks involving post-translational modification of proteins, and vi) the generation of sustained phosphorylation gradients by localized kinases and phosphatases. In my seminar I am going to talk about the potential generation of phosphorylation gradients in the cytoplasm and about their function in organizing cellular space and more specifically microtubules during mitotic spindle assembly.

During assembly of the mitotic spindle, microtubules grow preferentially towards chromosomes or are nucleated in their vicinity even in the absence of centrosomes. It is not yet entirely clear how these microtubules arise, whether they are nucleated off the chromatin or captured, but several lines of evidence suggest that they are in fact stabilized in the cytoplasm surrounding the chromosomes, independently of a physical contact with them [1]. This raises the question of how such a local stabilization is generated. I postulate that microtubule dynamics being regulated by phosphorylation-dephosphorylation reactions during mitosis, this local stabilization is due to the establishment around the chromosomes of a phosphorylation gradient of factors that regulate microtubule dynamics [2, 3]. Stabilizing factors, like MAPs bind preferentially to spindle microtubules rather than astral microtubules [4]. I suggest that this is due to a lower phosphorylation of MAPs around chromosomes. Moreover, a destabilizing factor, Stathmine/OP18, becomes phosphorylated and inactivated in frog egg extracts only when chromatin is added to the extract [5]. This suggests that the phosphorylation of this factor is regulated by soluble enzymes and other enzymes localized on chromosomes. This local inactivation of Stathmine/OP18 is probably one of the mechanisms involved in the preferential growth of microtubules towards chromosomes.

In living cells, there are examples of kinases localized to specific cellular domains that affect cellular morphogenesis. Orb, in Pombe, is one of them. I will discuss how phosphorylation gradients could also affect cellular morphogenesis in general.

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Proteins that recognize Microtubule ends may position organelles in cells.

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In a cell, organelles such as chromosomes or vesicles must position themselves in various places in the cell. Chromosomes position in the middle of the mitotic spindle using a microtubule-based mechanism. We have shown that kinetochores of S.cerevisae in vitro recognize the ends of microtubules. This suggests that chromosomes in vivo may position themselves in the spindle by recognizing the ends of microtubules. To test whether recognition of microtubule ends is a general mechanism for intracellular positioning, we have looked for proteins which recognize microtubule ends using a simple assay. Microtubules, either long or short, are added to a Xenopus extract. The microtubules are pelleted and the proteins binding to long versus short are compared. We have found that 1 protein, 215 KDa is enriched in short microtubules suggesting that it binds to microtubule ends. Western blots showed that this protein was XMAP215 originally purified by Gard and Kirschner. Mass Spec. showed that this protein with Tog, a protein overexpressed in human tumours. Western blotting shows that Tog is not localized to microtubule walls, but rather to membrane compartments in interphase and spindle poles in mitosis. This result suggests that end binding proteins could target the localization of membrane organelles to microtubule ends.

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Membrane rafts as morphogenetic devices

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In the prevailing view of cellular membrane structure, lipids in the bilayer function mainly as a solvent for membrane proteins. But in the fluid bilayer, different lipid species are asymmetrically distributed over the exoplasmic and cytoplasmic leaflets of the membrane. The lipids are also organized in the lateral dimension and impose more short- and long-range order than was previously recognized. This lateral organization results from preferential packing of sphingolipids and cholesterol into moving platforms, or rafts onto which specific proteins attach within the bilayer. We have proposed a model for membrane structure that describes the organization of these lipid microdomains and have presented evidence that proteins can selectively be included or excluded from these microdomains (Simons and Ikonen, 1997).

On the basis of our findings we have postulated the existence of two circuits in post-Golgi trafficking in mammalian cells (Simons and Ikonen 1997). The basal (non-raft) circuit transports membrane proteins with sorting signals in their cytoplasmic domains from the TGN to endosomes and the plasma membrane. The docking and fusion of this non-raft pathway is probably dependent on the SNARE mechanism. Endocytosis in this circuit is mediated by the clathrin-coated vesicle pathway to endosomes. The apical (raft) circuit, on the other hand, preferentially handles sphingolipid-cholesterol rafts and proteins associating with them. The mechanism of docking and fusion in this pathway is still unknown. The first station along biosynthetic route from the ER, where raft and non-raft components are sorted from each other to different routes, is the TGN. Endocytosis in the raft circuit consists of either caveolae internalizing cell surface components or the less well-defined clathrin-independent and actin-dependent route, the latter route probably being quantitatively the more important one.

An interesting example of how one of the two circuits is employed to polarize the cell surface is when fibroblasts become motile. Bergmann and Singer (1983) have demonstrated that the basolateral VSV-G protein becomes preferentially routed into the ruffling front of motile fibroblasts. This traffic is regulated by the basolateral Rab8 GTPase (Peränen et al. 1996). Obviously the prefential delivery of basolateral proteins such as integrins, involved in adhesive events important for cell locomotion, would ensure the continuous presence of proteins essential for forward movement. Another example is the polarization of the osteoclast during bone destruction (Salo et al. 1997). The surface facing the bone is more or less a lysosomal membrane, while digested bone products are delivered to the opposite cell surface pole by transcytosis.

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Surprisingly the plasma membrane away from the bone side is divided into two subdomains, one "apical" receiving the transcytosed cargo and the other "basolateral".

The polarized existence of apical and basolateral cognate routes in fibroblast/mesenchymal cells facilitates the generation of separate apical and basolateral cell surface domains during epithelial cell differentiation. Cells undergoing a mesenchymal-epithelial transition are known to respond to signals from the extracellular matrix or from neighbouring cells to form a basal pole (Drubin and Nelson 1996; Eaton and Simons 1995). If the formation of such a base were to lead to the assembly of a cytoskeletal scaffold fixing the apical pole, one could envisage that apical membrane proteins with preferential solubility for raft lipids bind to this pole. In this way a partial segregation of apical and basal cell surface domains could be achieved. Other apical proteins associating with rafts will move to the apex of the cell where sphingolipid-cholesterol rafts would tend to accumulate excluding basolateral proteins to the basal side of the cell. In fact, Rodriguez-Boulan et al. (1983) have observed that individual MDCK cells attaching to the substratum can support polarized budding of viruses; influenza virus forming apically and vesicular stomatitis virus basally. This experiment would suggest that partial segregation of the two membrane domains can be achieved without the installation of tight junctions as fences for blocking lateral diffusion.

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POSTERS

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The Cln3 cyclin as a key component of the mitosis-meiosis switch in budding yeast

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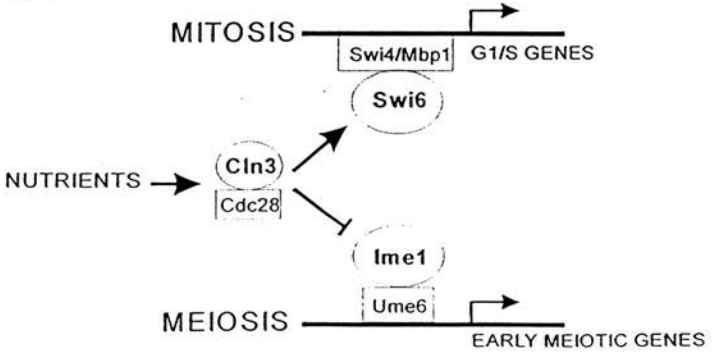
Diploid *Saccharomyces cerevisiae* cells are able to undergo meiosis under starvation conditions. Two different mechanisms are involved: (1) a diploid-specific control and (2) a poorly-understood nutrient sensing pathway. They ultimately regulate Ime1 activity, which is a key transcriptional activator of early genes during meiosis. While S-phase entry during the meiotic cycle depends on Ime1 by mechanisms not well characterized, mitotic DNA replication initiation is triggered by Cln3, the most upstream activator of the G_1/S transition. The possible role of Cln3 in the meiotic cycle has not been tested yet.

As in haploid cells, we have found that Cln3 levels are rapidly down-regulated by nutrient starvation in diploids cells well before they initiate meiotic DNA replication, suggesting that meiosis does not require the activity of this G_1 cyclin. Indeed, Cln3-deficient cells underwent meiosis more efficiently than wild type cells when, once arrested in G_1 by nutrient starvation, they were released into sporulation medium. Moreover, in partial starvation conditions where wild type cells arrest in G_1 but do not initiate a meiotic S-phase, Cln3-deficient cells entered very efficiently the meiotic S-phase after an initial G_1 arrest. In both cases, the higher sporulation efficiency of Cln3-deficient cells correlated with a concomitant increase in Ime1-dependent expression of early and middle meiotic genes. On the contrary, in G_1 arrested cells *CLN3* overexpression inhibited meiosis and greatly reduced Ime1-dependent gene expression. *CLB5* induction prior to meiotic DNA replication was Ime1 dependent, opposed to the mitotic DNA replication, where *CLB5* expression depends on Cln3, thus confirming the existence of different mechanisms to trigger the G_1/S transition during mitosis and meiosis. Preliminary observations indicate that the Cln3/Cdc28 kinase could down-regulate Ime1 activity at both transcriptional and post-translational levels.

Our results indicate that Cln3 is a meiotic repressor and we propose this G_1 cyclin as a key component of the molecular switch that makes mitosis and meiosis incompatible.



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ADENOVIRUS E1A TARGETS A NOVEL CDK2-REGULATED AND pRB-INDEPENDENT PATHWAY ESSENTIAL FOR G1-S PROGRESSION

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G1-specific cyclin-dependent kinases (CDKs) phosphorylate Retinoblastoma (pRb)-family proteins. While this may represent the essential function of CDK4 and CDK6 together with D-type cyclins, cyclin E/CDK2 complexes are believed to have at least one distinct function essential for G1-S progression. Viral oncoproteins such as adenovirus E1A, HPV E7 or SV40 Large T sequester pRb-family proteins, thereby bypassing the need for CDK4 and CDK6. We now show that E1A also reduces the requirement for CDK2 activity. Retroviral expression of E1A in rodent fibroblasts prevented G1 arrest induced by the CDK2 inhibitor $p27^{Kip1}$ and allowed long-term proliferation in the presence of ectopically expressed p27. Surprisingly, E1A neither bound, nor inactivated p27. Instead, p27 remained functional in E1A-expressing cells, inhibited the catalytic activity of cyclin E/CDK2 and cyclin A/CDK2 (including the E1A-associated kinase) and induced dephosphorylation of pRb. Moreover, E1A allowed proliferation of cells overexpressing p27 while c-Myc, which is known to induce a cellular p27-inhibitory activity, was only effective against physiological p27 levels. E1A also prevented G1 arrest by Roscovitine, a chemical inhibitor of CDK2. Altogether, these data show that E1A rescued growth by acting downstream of CDK2 function. Overcome of p27-induced arrest required two functional regions of E1A: the pRb-binding domain and a distinct amino-terminal domain spanning residues 26 to 35. Binding to p300/CBP was not required. Our data demonstrate that in addition to the pRb-family, E1A targets a novel cellular pathway(s) which comprises the additional essential effector(s) of CDK2 in G1-S control.

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Cell cycle role of the essential Ca2+/Calmodulin-dependent protein kinase II homologue in *Schizosacharomyces pombe*.

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The Ca^{2+} receptor protein calmodulin (CaM) is an important regulator of intracellular signalling in eukaryotic cells. Several studies suggest that Ca^{2+} and CaM are involved in controlling the G2/M transition in eukaryotic cells. Increased Ca^{2+} levels are associated with nuclear envelope breakdown and chromatin condensation. CaM is essential for cell-cycle progression at multiple points in the cell cycle including G2/M. However, little is known about the molecular basis for this requirement or the specific biochemical pathways regulated by CaM dependent enzymes.

CaM is known to regulate several different protein kinases and at least one protein phosphatase, it is possible that the role for CaM and CaM-regulated enzymes in the cell cycle regulation involves modulation of the phosphorylation state of key cell cycle regulatory proteins. Consistent with this prediction, previous studies have suggested that the multifunctional CaM-dependent protein kinase II (CaMKII) is required for the G2/M transition. Microinjection of a constitutively active truncated form of CaMKII into *Xenopus* oocytes arrested at metaphase of meiosis II, induces APC mediated cyclin degradation and $p34^{cdc2}$ inactivation. In contrast, overexpression of a constitutively active form of mammalian CaMKII in both mouse C127 cells and *Schizosaccharomyces pombe* results in a G2 arrest. Thus, the existing evidence pointed to a role for an ordered phosphorylation reaction initiated by CaMKII in the regulation of entry into and progression through mitosis/meiosis.

We have identified the CaM-dependent kinase II homologue in *S.pombe* (pCaMKII). The amino acid sequence contains all the consensus domains present in other serine/threonine protein kinases and it has putative autophosphorylation sites present in the regulatory domain of CaMKII that upon phosphorylation are responsible for the generation of CaM-independent kinase activity. The predicted amino acid sequence is most closely homologous to the rat and *A. nidulans* CaMKIIs and, to a lesser extent, to the *Xenopus laevis* and *S. cerevisiae* CaMKIIs.

Gene disruption experiment revealed that pCaMKII gene function is essential for vegetative growth of *S. pombe*.

pCaMKII binds CaM in Ca^{2+} dependent conditions. This result together with the ability of pCaMKII to phosphorylate MBP specifically in presence of Ca2+/CaM suggest the existence of a regulatory CaM-binding domain capable to modulate autophosphorylation and activation of the kinase.

Overexpression of full length kinase does not block mitosis, as it does the expression of the vertebrate constitutively active form of pCaMKII, but cell cycle is longer in this cells compared with wild type cells. Construction and expression of a constitutively active form of pCaMKII will allow us to see whether pCaMKII has a drastic effect in G2/M trasition.

Initial studies of endogenous CaMKII fused to the green fluorescence protein in asynchronous S. pombe cultures have shown CaMKII located in the cytoplasm of few cells. This preliminary result together with the analysis of CaMKII mRNA suggest that CaMKII is cell cycle regulated.

Further studies will be done to achieve the main objective, to elucidate the role of CaMKII in the regulation of entry into and progression through mitosis/meiosis.

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THE CONCOMITANT ALTERATION OF THE CYCLIN D-CDK4-PRB AND CYCLIN E/A-CDK2 PATHWAYS IN TUMOR CELLS CONFERS HIGH RATE OF CELL PROLIFERATION

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We have analyzed the alterations of the key proteins of the G1/S cell cycle regulatory machinery in a panel of 8 human tumor cell lines (4 from pancreas and 4 from colon) and in 8 human pancreatic tumors (ortotopically transplanted to atimic mice).

Specifically, we analyzed the levels of cyclin D1, cyclin D3, cdk4, p16, p21, pRb, cyclin E, cyclin A, cdk2, p27, cyclin H, cdk7 and cdc25A by western blotting.

Conclusions: Three main conclusions can be obtained from these studies:

1) All the cell lines and xenografic tumors have at least one alteration in the cyclin D-cdk4-pRb pathway (more of the cells and tumors having more than one alteration). The alterations lead to an hyperactivation of the pathway and consequently to an upregulation of the expression of E2F-depending genes as cdc2, p107 and PCNA. All the members of the pathway with the exception of cyclin D1 (cdk4, cyclin D3, pRb, p16 and p21) have the same probability to be altered (around 50%).
2) Most of the tumor cell lines and tumors (62%) have also alterations in the cyclin E/A-cdk2 pathway (All the members of this pathway have more or less the same probability to be altered
3) The tumor cell lines showing concomitant alterations of these two pathways show a higher rate of proliferation than those showing only alterations in the cyclin D-cdk4-pRb pathway.

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Functionally homologous DNA replication genes in fission and budding yeast

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A general feature of all eukaryotes is that genome replication alternates with cell division. A model derived from experimental work in animal and yeast cells suggests that two different signals are required for DNA replication. One makes replication origins competent for the initiation of DNA synthesis and the other induces initiation on competent origins. This second signal is provided by the S phase promoting factor (SPF) one of the multiple forms of cyclin dependent kinases (CDKs). A complex (ORC) of six different proteins is bound to *Saccharomyces cerevisiae* replication origins throughout the cell cycle. Additional factors are recruited to the ORC complex at the G1/S transition when replication origins are going to be activated. The periodically expressed Cdc6p protein associates with ORC to promote DNA replication. Moreover, pre-replicative origins competent for initiation are thermolabile in a cdc6-1 temperature sensitive mutant background suggesting that transition from a non-competent post-replicative to a competent pre-replicative status relies on *CDC6*. The *cdc18+* gene of the fission yeast *Schizosaccharomyces pombe*, a putative homolog of *CDC6*, is involved in the initiation of DNA replication as well as in coupling S phase to mitosis. Our results show that the *Saccharomyces cerevisiae CDC6* gene complements both the initiation and the checkpoint defects associated to the lack of *cdc18+* when expressed in fission yeast. Cdc6 protein interacts *in vivo* with Cdc2 kinase complexes, and overexpression of Cdc6 in fission yeast induces multiple rounds of S-phase in the absence of mitosis and cell division, as well as Cdc18 does in *S.pombe* cells. This *CDC6*-dependent continuous DNA synthesis phenotype is independent of the presence of a functional *cdc18+* gene product. Our data show that *CDC6* and *cdc18+* are functional homologues and also support the idea that controls restricting genome duplication might diverge in fission and budding yeast.

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CYCLIN E DETERMINES THE ESTABLISHMENT OF ENDOREPLICATION IN MEGAKARYOBLASTIC CELLS

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The strict regulation needed for the proper succession of S and M phases is likely to be altered in cells in which endoreplication occurs. During haemopoiesis the megakaryocyte specifically undergoes endoreplication, and we showed previously that in the megakaryoblastic cell lines HEL and MEG01 the process is paralleled by maintained levels of G1/S cyclins and down-regulation of the mitotic activator cdc25C. The megakaryoblastic cell line K562, however, progresses to mature megakaryocytic forms in the presence of TPA but does not undergo endoreplication. To analyse the role of cyclin E in endoreplication, we derived cyclin E over-expressing K562 clones; these exhibited no phenotypic changes under normal growth conditions but they responded to TPA by entering endoreplication. The establishment of endoreplication correlated with maintenance of cyclin A levels, which are down-regulated by TPA in the parental K562 cells. Transient transfection experiments revealed that in endoreplicating HEL and MEG01 cells the cyclin A promoter is not affected by the presence of TPA, whereas it is repressed in the non-endoreplicating K562 cells; however, both constitutive or transient co-expression of cyclin E in K562 relieved this repression. Furthermore, transient expression in K562 of cyclin A as well as cyclin E was found to determine the appearance of polyploid cells in the presence of TPA. We conclude that cyclin E is directly involved in the establishment of endoreplication in megakaryoblastic cells by maintaining cyclin A expression in the presence of differentiative signals.

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The rum1p promotes proteolysis of the mitotic B-cyclin cdc13p during G1 of the fission yeast cell cycle.

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One major regulator of G1 progression in fission yeast is the *rum1+* gene product We have shown that rum1p is crucially involved in keeping the mitotic cdc2/cdc13 kinase at low levels in G1 acting as a CDK inhibitor. As well as being a CKI, rum1p could have a second role targeting the cdc 13p for proteolysis in G1. In fission yeast, early G 1 cells have low levels of cdc13p. These undetectable levels are not brought about by regulating transcription or translation of cdc 13. These observations suggest that proteolysis of cdc 13 is important in keeping low cdc2 kinase activity in G1 cells. In the absence of rum1, cdc13p proteolysis in G1 is reduced to a level which is insufficient to counteract the continuing translation of cdc13p. As a consequence, the level of cdc13 remains high in cells lacking rum1. Another observation that suggests rum1p might act as an adapter specifically -targeting cdc13p for proteolysis comes from studies on pheromone induced G1 arrest. In pheromone treated cells, rum1p and cyclosome dependent proteolysis of B type cyclins are required to bring about the G1 arrest. In cells lacking rum1, cdc13p remains at high levels in the presence of pheromone, however, the B-type cyclin cig2 is still down regulated. In a nuc2 mutant (homologous to the budding yeast CDC27), both B-type cyclins are at high levels. These observations suggest that rum1p is required for cyclosome mediated cdc13p proteolysis in response to pheromone and cig2p is destroyed by the cyclosome in a rum1 independent manner.

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"Phosphatidic acid and ceramide, two antagonistic lipid second messengers crucial for the T lymphocyte fate"

Stimulation via the interleukin-2 receptor induces CTLL-2 cells to transit through the cell cycle. On the other hand, deprivation of interleukin-2 carries CTLL-2 cells to a transient cell cycle arrest followed by apoptosis. In the proliferative, quiescent and apoptotic processes, phosphatidic acid and ceramide have opposite effects. The actions of these lipids have been examinated in detail.

Previous experiments have demostrated that interleukin-2 receptor ligation induces phosphatidic acid production through activation of the α isoform of diacylglycerol kinase (DGKα). We demonstrate that treatment of CTLL-2 cells with a DGKα inhibitor prevents the entry of the cells into S phase. To further demonstrate the role of phosphatidic acid in the initiation of the proliferative signal delivered by IL-2, we show that following cell cycle arrest by inhibition of DGKα, addition of phosphatidic acid can reconstitute the proliferative response. The analysis of the proteins induced following IL-2 addition demonstrates that inhibition of DGKα activity has a profound effect on the induction of proto-oncogenes c-myc, c-fos and c-raf by IL-2 and on the hyperphosphorylation state of pRb. On the other hand, expression of bcl-2 and bcl-x_L are not affected following DGKα inhibition.

Increases in ceramide have been related to the onset of apoptosis, terminal differentiation and growth supression. In this study, addition of exogenous C_2-ceramide to CTLL-2 cells is found to block IL-2 cell cycle entry, as well as the apoptosis triggered by IL-2 deprivation. The protective effect of C_2-ceramide is achieved only at short times following cytokine deprivation and is related to the inhibition of bcl-x_L degradation and the induction of a G0-arrest of cells. The same treatment at longer times when, as we demonstrate, ceramide is produced physiologically, enhances cell death by apoptosis. The dual effect of ceramide in protecting from or inducing apoptosis is further discussed.

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MPF shuttles between the nucleus and the cytoplasm by a leptomycin B-sensitive pathway

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In eukaryotes mitosis is initiated by MPF (M phase Promoting Factor), composed of B-type cyclins and their partner protein kinase, CDK1. In animal cells, MPF is cytoplasmic in interphase and is translocated into the nucleus after mitosis has begun, after which it associates with the mitotic apparatus until the cyclins are degraded in anaphase. We have used a fusion protein between human cyclin B1 and Green Fluorescent Protein (GFP) to study this dynamic behaviour in real time, in living cells. We found that when we injected cyclin B1-GFP, or cyclin B1-GFP bound to CDK1 (i.e. MPF), into interphase nuclei it is rapidly exported into the cytoplasm. Cyclin B1 nuclear export is blocked by leptomycin B, an inhibitor of the recently identified export factor, exportin 1 (CRM1). The nuclear export of MPF is mediated by a novel nuclear export sequence on cyclin B1, and an export defective cyclin B1 accumulates in interphase nuclei. Thus we can conclude that during interphase MPF constantly shuttles between the nucleus and the cytoplasm. These studies indicate that cyclin B1/CDK1 could respond to signals in both the nucleus and the cytoplasm, and therefore coordinate their entry into mitosis.

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Cyclin expression in response to stress conditions in *Saccharomyces cerevisiae*

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Heat shock or oxidative stress cause a transitory cell cycle arrest at G1 in *S. cerevisiae* (1,2). We have observed that moderate heat shock (shift to 37°C), oxidative stress (KCl 0.5M) or oxidative stress (menadione 0.2 mM), which are all of them conditions that do not affect cell viability, lead to transitory downregulation of *CLN1*, *CLN2* and *CLB5* expression but not of *CLN3*, in asyncronous cultures. This decrease on cyclin expression is partially supressed in *swi6* mutants. Levels of Cln3p increase concomitantly with the decreased expression of the above cyclin genes, a significant fraction of the protein being hyperphosphorylated. Downregulation of *CLN2* expression was confirmed in cultures syncronized by nitrogen starvation. Cell so arrested in G1 (3) were refed and after a time, they were heat- or osmotically-shocked. Stressed cells displayed a 20 min delay in regaining *CLN2* expression with respect to non-stressed cells. A similar delay was observed in the appearence of 2n cells as determined by FACS analysis. These results indicate that different moderate stresses cause a pre-Start G1 cell cycle arrest. Nitrogen-starved *swi6* mutant cells syncronously arrest with high *CLN2* mRNA levels. When stressed in the same conditions they maintain constitutively high transcript levels, discarding the existance of a *SWI6*-dependent inhibitory mechanism on the activated SBF factor responsible of G1 cyclin expression.

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Positive and negative signalling converging on E2F in B-lymphocytes

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IgM and CD40 are surface receptors important for B-lymphocyte development. Using a mouse B lymphoma cell line, WEHI-231 as a model system for immature B-lymphocytes to examine the negative and positive proliferative signals, we identified the transcription factor E2F as a downstream target for IgM and CD40 signalling. We demonstrated that E2F1 expression was repressed by IgM signalling and upregulated by CD40 mediated signal and showed that this was regulated transcriptionally through the E2F-binding site of its promoter. Consistent with previous observations, we showed that this regulation of E2F1 was controlled by the phosphorylation status of the p130 protein. We also obtained data implicating cyclin D2 and p27 as important regulators of p130 phosphorylation and consequently E2F1 expression.

We have also extended these studies to normal mouse primary B-lymphocytes and demonstrated similar signalling pathways exist, though in mature B-lymphocytes the IgM signals promote rather than inhibit proliferation. To also study the functional significance of these signalling molecules implicated in controlling proliferation during B-lymphocyte development, we used 'knock-out' mice lacking these molecules as a source of primary B-lymphocytes for proliferation experiments. We have now acquired preliminary data indicating cyclin D2 is essential for B-lymphocyte development as mature B lymphocytes fail to proliferate in response to IgM stimulation. We are now investigating the upstream signalling pathways regulating cyclin D2 and p27 expression in B-lymphocytes.

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The RanBP1 gene and control of cell cycle progression

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The small nuclear GTPase Ran is the central component of a signalling network that regulates several functions, including cell cycle progression, mitotic entry and exit, nucleocytoplasmic transport and nuclear structure. We have identified and cloned the murine gene encoding Ran-binding protein 1 (RanBP1), a major molecular partner of Ran. Unlike most Ran-interacting partners that are constitutively active, transcription of the *RanBP1* mRNA is repressed in non proliferating cells and is up-regulated in S phase. We have altered the correct pattern of RanBP1 expression and have found that this disrupts the orderly execution of the cell division cycle. Major defects include inhibition of DNA replication in cells re-entering the cycle from G0, impairment or delay of mitotic exit in cells released from metaphase arrest, and failure of chromatin decondensation during the mitosis-to-interphase transition. These defects are reminiscent of defects caused by RCC1 loss of function.

In the attempt to depict the molecular basis of the defects that accompany RanBP1 deregulated expression, we have begun to investigate the interactions that take place between components of the Ran network during an ordinary cell cycle. Western immunoblotting and immunofluorescence (IF) studies in synchronously cycling cells show that RanBP1 levels are up-regulated from S to M phase, unlike Ran and RCC1 which are both steadily expressed throughout the cell cycle. IF studies also depicted specific subcellular localizations of each of these components in distinct phases. Thus, these studies suggest that specific interactions among Ran, RCC1 and RanBP1 are established during cell cycle phases, reflecting both cell phase-specific expression and compartimentalization of specific components. Induction of forced expression of the *RanBP1* gene alters the phase-specific balance among members of the Ran network, suggesting that the unbalance interferes with the function of cellular targets whose activity is required for further cell cycle progression and completion of the mitotic division.

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Apoptosis in proliferating neuroepithelial cells during retinal neurogenesis

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A precisely coordinated regulation of cell proliferation, differentiation and survival during neural development is necessary for the generation of the highly complex vertebrate central nervous system. While the role of programmed cell death (PCD) is clearly established by the classical neurotrophic theory for connecting neurons, much less is known about how proliferating neuroepithelial cells are affected by PCD. We had previously reported that insulin and IGF-I, acting as embryonic growth factors, appear to promote both cell proliferation and differentiation in chick embryonic neuroretina (Hernández-Sánchez et al., 1995. Proc. Natl. Acad. Sci. USA. 92, 9834). Since proliferation and differentiation are mutually exclusive for neuroepithelial cells, we have explored whether attenuation of apoptosis by insulin is underlying both effects. Apoptosis indeed occurs during retinal neurogenesis, following a very defined topological pattern that can be drawn in whole mount retina as "isothanas". Insulin apparently stimulated proliferation of E5-E6 chick embryo neuroretina in organoculture, decreasing the cell cycle duration as determined by cell flow through S-phase. In parallel, a reduction of apoptosis was observed by multiple criteria, including presence of pyknotic nuclei, TUNEL staining and ELISA determination of free nucleosomes. IGF-I and proinsulin showed similar effects. Giving a [^{3}H]thymidine pulse in ovo followed by chase in organoculture, in the presence or the absence of insulin, it was possible to determine that insulin decreased the degradation of labeled DNA to nucleosomes. Furthermore, apoptosis seems to affect mainly to cells that recently went throughout a S-phase. These data altogether suggest the existence of a balance between proliferation and apoptosis in the neuroepithelial cells, regulated by insulin, present endogenously in the embryonic retina at this stage.

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Cell Cycle Calcium Signalling During Early Development

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During the first cell cycle of sea urchin embryos transient increases in cellular calcium are detected prior to nuclear envelope breakdown (NEB), anaphase onset and during cytokinesis. In each case the transient increase in calcium is necessary for further progression through the cell cycle. Data indicating that the anaphase-related calcium signal triggers the separation of chromatids has recently been published[1].

Calcium signalling during early *Drosophila* embryogenesis has not been studied previously. By injecting the fluorescent dye calcium green dextran into *Drosophila* embryos we have been able to detect a transient increase in cellular calcium at the telophase/interphase boundary during division cycles 8 to 13. This peaks in mid-interphase and falls immediately before NEB. The increase in cytosolic calcium is accompanied by the 'relaxation' of the yolky interior and the movement of the peripheral cytoplasm from the poles towards the centre of the egg. The increase in calcium occurs in the form of as two slow moving (approx. 0.4μm/s) waves originating near to both the posterior and anterior poles and meeting at the equator. During the syncytial blastoderm stages mitosis occurs progressively in the nuclei on the embryonic surface as a wave front[2]. The areas of increased calcium always contain interphase nuclei. The final calcium wave to move through the embryo is detected immediately after mitosis 13.

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THE *Schizosaccharomyces pombe* PKC HOMOLOGS pck1 AND pck2 ARE rho1p TARGETS AND REGULATE THE (1-3)ß-GLUCAN SYNTHASE MEMBRANE COMPONENT.

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In a previous study we have demonstrated that *S. pombe* rho1p GTPase activates the glucan synthase and is involved in actin organization but other downstream effectors of rho1p remain to be established. In *S. cerevisiae* this GTPase also physically interacts and activates PKC1 in a GTP-dependent manner (Kamada *et al.*, 1996). Since components such as GTPases and protein kinases are conserved among both yeasts, we study here the possible relationship of rho1p with pck1 and pck2 and the role of this molecules in the cell wall biosynthesis. *pck1+* and *pck2+*, the two fission yeast PKC homologues, were isolated by Toda et al. (1993). Both genes share overlapping roles in cell viability and partially complement each other. *Δpck1* does not have a clear phenotype whereas *Δpck2* cells have a defect in cell wall construction. Cells are bended or rounded and show and abnormally thin cell wall hypersensitive to lytic enzymes (Toda et al., 1996).

We show in this study that rho1p physically interacts with both pck1 and pck2 and we have mapped the interaction. The level of (1-3)ß-D-glucan synthase activity from the *Schizosaccharomyces pombe* *Δpck2*, *Δpck1* and *pck2-8* mutant strains was only slightly different from the wild type. On the other hand, all *Δpck2*, *Δpck1* and pck2-8 mutants were hipersensitive to the antifungal drug Papulacandin B and *Δpck1* was also hipersensitive to Calcofluor and Echinocandin, suggesting that they have a defective cell wall. Overexpression of the *pck2*$^{+}$ gene in wild type *S. pombe* cells doubled the (1-3)ß-D-glucan synthase activity. When this activity was separated into detergent soluble and insoluble fractions and reconstituted, the increased caused by pck2 overexpression was exclusively detected in the insoluble fraction. Moreover, when the activity was reconstituted using the soluble fraction of cells overexpressing the *rho1*G15V constitutively active mutant, and the insoluble fraction of cells overexpressing *pck2+*, the (1-3)ß-D-glucan synthase level was six times higher than the wild type, indicating that the effect of those two proteins on the enzyme is additive. Overexpression of *pck2+* caused aberrant morphology and a general increase in the cell wall biosynthesis that was more pronounced in the β-glucans.

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PHOSPHORYLATION OF HUMAN CDC6 BY CYCLIN A/CDK2 REGULATES ITS SUBCELLULAR LOCALISATION

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The cyclin-dependent kinases (CDKs) are essential for regulating key transitions in the cell cycle, including the initiation of DNA replication, mitosis, and prevention of rereplication. In order to unravel the molecular mechanisms regulating cell cycle progression, it is essential to identify the substrates for the CDKs and to understand the regulatory function of CDK phosphorylation. Recently, we and others isolated the human homologue, of *S. cerevisiae* Cdc6p, *S. pombe* Cdc18p, and *X. laevis* XlCDC6 that all are essential for the initiation of DNA replication. Here we demonstrate that human CDC6 (hCDC6) is phosphorylated by CDKs, and that hCDC6 interacts specifically with the active Cyclin A/CDK2 complex *in vitro* and *in vivo*, but not with Cyclin E and Cyclin B kinase complexes. The binding domain in hCDC6 was mapped to a N-terminal Cy-motif, which is similar to the cyclin binding regions in $p21^{WAF1/sdi1}$ and CDC25A. The *in vivo* phosphorylation of hCDC6 was dependent on three N-terminal CDK consensus sites, which were mutated to either alanine (A) or aspartic acid (D). Immunostainings showed a dramatic difference in subcellular localisation of hCDC6 in cells expressing either the wild type (WT), the triple A mutant (AAA) or the triple D mutant (DDD). Whereas the WT protein is located both in the nucleus and in the cytoplasm, the AAA mutant is mainly nuclear, and the DDD mutant is located only in the cytoplasm, and consistent with this notion, we found that the subcellular localisation of hCDC6 is cell cycle regulated. In G1, hCDC6 is nuclear and becomes cytoplasmic around the G1/S transition, when Cyclin A/CDK2 is activated. In agreement with that hCDC6 phosphorylation is specifically mediated by Cyclin A/CDK2, we show that ectopic expression of Cyclin A, and not of Cyclin E, leads to rapid delocalisation of hCDC6. Based on our data we suggest that the specific phosphorylation of hCDC6 by Cyclin A/CDK2 prevents rereplication during S and G2.

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Thyroid hormone receptor expression induces cell cycle withdrawal in retroviral-infected cells accompanied by an increase in the inhibitor p27/ Kip1.

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Thyroid hormone plays a key role in the correct development of the central nervous system. Previous studies indicated that thyroid hormone can control the proliferation and differentiation of the nerve cells, and these effects can be mediated by the different thyroid hormone receptors (T3R) isoforms. Thus, thyroid hormone receptor α isoforms are expressed in oligodendrocyte progenitor cells. Differentiation to oligodendrocytes is associated with the expression of the thyroid hormone receptor β isoform, suggesting that during this event some thyroid hormone effects could be isoform-specific.

In order to established the potential role of each thyroid hormone receptor isoform in the exit from the cell cycle that is required for differentiation, we have ectopically expressed α1 and β1 isoform by means of retroviral vectors. We show that expression of the T3R is sufficient to induce growth arrest in Swiss 3T3 fibroblasts. This effect is isoform-dependent being the β1 isoform more potent that α1. Addition of thyroid hormone decreases further the growth rate of the receptor-expressing clones. Cell cycle withdrawal is accompanied by an increase in the amount of the cyclin-dependent kinase (Cdk) p27/kip1 inhibitor that correlates with the thyroid hormone receptor that has being expressed. Furthermore, thyroid hormone addition increases the p27 protein half-life resulting in a delayed cell cycle re-entry. These results demonstrate that proliferation control by thyroid hormone is receptor isoform-specific and its regulation is differentiation independent.

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Role of UEV-1 in Cell Cycle Progression

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UEV (Ubiquitin Enzyme Variant) has been defined as a family of proteins with similarities to the ubiquitin conjugating enzymes, but lacking their enzymatic activity (Sancho et al, 1998). These proteins are highly conserved in phylogeny from yeast to mammals. Constitutive expression of exogenous human UEV in HT-29 M6 cells, inhibited their capacity to differentiate upon confluence and caused both the entry of a larger proportion of cells in the division cycle and an accummulation in G2/M. This was accompanied with a profound inhibition of the mitotic kinase CDK1 (Sancho et al, 1998). Disruption of UEV in *S. cerevisiae* rendered the cells more sensible to DNA damaging agents. FACS analysis indicates that ΔUEV mutant cells subjected to DNA damaging agents irreversibly arrest with a G2/M DNA content (Thomson et al, 1998). These results implicate UEV proteins in the regulation of cell cycle progression through G2/M.

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Identification and characterization of Smad interacting proteins.

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Smad proteins have been identified as cytoplasmic mediators of transforming growth factor-β (TGF-β) family signaling pathways. TGF-β induces serine phosphorylation of Smad2 and Smad3. This phosphorylation leads to oligomerization with a common mediator Smad4, and to nuclear translocation of the Smad complex. Smad2/3-Smad4 complexes are able to activate the expression of several TGF-β responsive genes by interacting with transcription factors. In this way, the Xenopus DNA binding protein, FAST1 has been shown to interat with Smad complexes, and this interaction is required for activation of the Mix2 gene. We have tested that FAST1 can bind to the carboxy-terminal domain of Smad2 and Smad3 in the yeast two hybrid system. In order to identify other proteins involved in TGFβ signaling, we have searched for Smad-binding proteins by two hybrid screening of Smad2, Smad3 and Smad4 as baits. We used a HIS3 reporter with 3-amino-1,2,4-triazole to overcome the basal transcriptional activation of the Smad baits. Among the positive clones interacting with Smad2 and Smad3, we have found smad4, which has served as a positive control to validate the system. In addition, we have identified potential DNA binding proteins with zinc finger domains as well as several other novel proteins that interact with Smad2 and Smad3. We are currently characterizing these interactions and their roles in TGF-β signaling.

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P27^{KIP1} IS ANOMALOUSLY EXPRESSED IN DIFFUSE LARGE CELL LYMPHOMAS, AND IS ASSOCIATED WITH ADVERSE CLINICAL OUTCOME

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Cell cycle progression is regulated by the combined action of cyclins, cyclin-dependent kinases (CDKs) and kinase inhibitors (CDKI). P27^{KIP1} is a universal inhibitor involved in G1 arrest in response to certain agents known to inhibit cell cycle progrssion, such as TGF-beta and cell-cell contact.

The aim of this study was to analyse p27^{KIP1} expression in cases of Diffuse Large Cell Lymphoma, correlating it with the proliferative index, clinical outcome, p53 status and Cyclin D3 expression.

MATERIAL AND METHODS: In a group of 130 cases with known clinical course, p27/^{KIP1}, ki67(proliferation marker), p53, p21 ^{WAF1} MDM2 and Cyclin D3 were studied by inmunohistochemistry techniques in paraffin-embedded tissue. Stadistical analysis was performed in order to ascertain which clinical and molecular variables may influence the outcome. The relationships between p27^{KIP1} expression, the proliferation index, p53 status and Cyclin D3 expression were also tested.

RESULTS: An abnormally high expression of p27^{KIP1} was detected in a small group of lymphomas. The overall correlation between p27^{KIP1} and ki67 showed no significant relationship between these two parameters, differing from observations in reactive lymphoid tissues.

Analysis of p27^{KIP1} expression in lymphomas shows that a high level of p27 expression is an adverse prognostic marker in univarient and multivarient analysis.

This p27^{KIP1} expresión is significantly associated with the inactivated supressor pathway of p53 (p53+, p21-, MDM2 -).

There is also a clear relationship between high levels of Cyclin D3 and p27^{KIP1} expression.

DISCUSSION: The results described show that p27^{KIP1} expression is anomalous in a group of Difuse Large Cell Lymphomas, associated with adverse clinical significance. This high p27^{KIP1} expression paralled with high expression of Cyclin D3 suggest that p27^{KIP1} is sequestered by the Cyclin D3/CDK4 complex, impeding the action of p27 ^{KIP1} on Cyclin E/CDK2, in a group of high grade lymphomas.

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A screen for genes involved in growth regulation of imaginal discs.

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During the development of Drosophila imaginal discs, growth and patterning take place at the same time: cells continue to proliferate while being instructed about their prospective fate. As a step toward understanding how patterning and proliferation are coordinated, we carried out a screen for genes involved in growth control of imaginal discs.

Proliferation in discs is a regulative process. Changes in the growth rate of a clone of cells is compensated for by neighboring cells, generally resulting in a wing or leg of normal shape and size. Consequently, genes regulating proliferation might be missed in conventional genetic mosaic screens. In contrast genes whose expression influences growth rate might result in a detectable phenotype when overexpressed in a large region of the disc. The screen was based on overexpression of random genes. 2300 lines carrying random P element insertions containing a UAS response element were crossed to flies expressing Gal4 under control of the omb promotor (ombGal4) and the wings and legs of the progeny were examined for growth and pattern abnormalities (Rorth et al, 1998). If the P element is inserted in the right orientation at the 5' end of a gene, the gene will be overexpressed in the omb domain.

This report will focus on a group of lines which caused changes in proliferation of the discs without obvious changes in patterning. Other lines alter patterning and consequently alter growth. 5 lines reduce the size of the region where they are expressed. One of these corresponds to yan/pokuri, an inhibitor of EGF receptor signaling, which is known to be required for the growth of the disc. In addition one line was identified that increases growth of the region in which it is expressed.

These results suggest that the screen has the potential to find important genes implicated in growth control. We will present a comparison of how growth is altered by genes of this class when compared to genes that affect patterning (using a novel method to monitor region specific growth). Preliminary results on the molecular characterization and on phenotypic analysis of lack of function mutation will be presented for a subset of the new genes.

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