Decoding the links between mitosis, cancer, and chemotherapy: The mitotic checkpoint, adaptation, and cell death

Beth A.A. Weaver[1] and Don W. Cleveland[1,*]

[1]Ludwig Institute for Cancer Research and Department of Cellular and Molecular Medicine, University of California at San Diego, La Jolla, California 92093
*Correspondence: dcleveland@ucsd.edu

Disrupted passage through mitosis often leads to chromosome missegregation and the production of aneuploid progeny. Aneuploidy has long been recognized as a frequent characteristic of cancer cells and a possible cause of tumorigenesis. Drugs that target mitotic spindle assembly are frequently used to treat various types of human tumors. These lead to chronic mitotic arrest from sustained activation of the mitotic checkpoint. Here, we review the linkage between the mitotic checkpoint, aneuploidy, adaptation from mitotic arrest, and antimitotic drug-induced cell death.

Almost 100 years ago, Theodor Boveri, working with sea urchin embryos containing aberrant numbers of spindle poles, observed that the progeny produced contained abnormal numbers of chromosomes, a condition described as aneuploidy (Boveri, 1914). At that time it was already well known that aneuploidy was a common characteristic of tumors, and Boveri proposed that aneuploid progeny produced from a disrupted mitosis become the progenitor cells of tumors. This original hypothesis remains untested. Nevertheless, a link between mitosis, aneuploidy, and cancer has been widely assumed.

Contrary to its potential for initiating tumorigenesis, mitosis has also served as a successful antitumor target. Indeed, drugs that arrest cells in mitosis, known as antimitotics, are common treatments for a variety of human tumors, including breast, ovarian, and non-small-cell lung cancer. However, the mechanism linking long-term mitotic arrest to cell death has remained almost completely unexplored. The paper published in this issue by Tao and colleagues (Tao et al., 2005) represents some of the first evidence on the clinically relevant linkage between mitotic arrest, the mitotic checkpoint whose action is responsible for that arrest, and cell death. In light of this evidence, we review here what is currently known about the mitotic checkpoint and its role in tumorigenesis and cell death, and highlight some of the fundamental questions that remain unanswered.

The mitotic checkpoint: The primary defense against aneuploidy

The mitotic checkpoint, also known as the spindle assembly checkpoint, is the major cell cycle control mechanism in mitosis. It is responsible for the production of genetically identical daughter cells by ensuring accurate chromosome segregation. Proper chromosome segregation requires that one copy of each pair of replicated sister chromatids be delivered to each daughter cell. This is accomplished by organizing the sister chromatids on a bipolar mitotic spindle composed of microtubules (Figure 1). The chromosomes connect to spindle microtubules through their kinetochores, protein-rich structures that assemble and disassemble every mitosis at sites of centromeric DNA, located at the primary constriction of the chromosome. Unattached kinetochores generate diffusible checkpoint complexes that comprise a "wait anaphase" signal, which delays the irreversible process of sister chromatid separation until each and every kinetochore has become productively attached to the mitotic spindle. This ensures the faithful segregation of sister chromatids and the prevention of aneuploidy.

At a molecular level, the mitotic checkpoint prevents advance to anaphase by producing an inhibitor(s) of the anaphase-promoting complex (APC). The APC is an E3 ubiquitin ligase which ubiquitinates mitotic substrates whose subsequent proteosome-mediated destruction is necessary for anaphase onset. APC inhibition is accomplished by recruiting checkpoint proteins, including Bub1, BubR1, Bub3, Mad1, and Mad2 to unattached kinetochores (Figure 2A). There, they are converted into one or more inhibitors of Cdc20, the specificity factor that APC requires to recognize mitotic substrates, including securin and cyclin B. The identity of the in vivo inhibitor(s) remains unknown, but may include activated forms of Mad2 or BubR1, or a complex of Cdc20, Mad2, BubR1, and Bub3 (Figures 1B and 2A) (Fang, 2002; Fang et al., 1998; Sudakin et al., 2001; Tang et al., 2001). After all kinetochores have properly attached (metaphase; Figure 1C), signal generation is silenced and the APC^{Cdc20} inhibitors decay through an ill-defined mechanism that may include the action of the Mad2 binding factor Cmt2 (Habu et al., 2002). APC^{Cdc20}-mediated ubiquitination of securin leads to activation of its binding partner separase, which cleaves the cohesins that maintain the linkage between sister chromatids, leading to sister chromatid separation and anaphase onset (Figure 1D). Ubiquitination and degradation of cyclin B inactivates Cdk1, thereby permitting exit from mitosis (reviewed in Wasch and Engelbert, 2005). In this fashion, the mitotic checkpoint prevents aneuploidy by permitting unattached kinetochores on chromosomes that would be missegregated to delay the irreversible transition from metaphase to anaphase until they become appropriately attached.

The mitotic checkpoint was initially recognized 15 years ago in experiments using antimitotic drugs that depolymerize microtubules (Hoyt et al., 1991; Li and Murray, 1991). These microtubule poisons cause all kinetochores to become unattached and, therefore, a maximal mitotic checkpoint signal is generated. In the succeeding years, testing for the ability to arrest in response to microtubule poisons has been commonly used as the sole test for checkpoint competence. This approach fueled a view still held by some that the checkpoint is either "on" or "off," depending on whether or not cells accumulate in mitosis in response to spindle disruption. This view is incorrect. The primary role of the mitotic checkpoint is to protect against misseg-

ON THE ROAD TO CANCER: ANEUPLOIDY AND THE MITOTIC CHECKPOINT

Geert J. P. L. Kops, Beth A. A. Weaver‡ and Don W. Cleveland‡*

Abstract | Abnormal chromosome content — also known as aneuploidy — is the most common characteristic of human solid tumours. It has therefore been proposed that aneuploidy contributes to, or even drives, tumour development. The mitotic checkpoint guards against chromosome mis-segregation by delaying cell-cycle progression through mitosis until all chromosomes have successfully made spindle-microtubule attachments. Defects in the mitotic checkpoint generate aneuploidy and might facilitate tumorigenesis, but more severe disabling of checkpoint signalling is a possible anticancer strategy.

ADENOMATOSIS POLYPOSIS COLI
Tumour-suppressor protein that, in a mutated, defective form, causes familial adenomatous polyposis (FAP), a rare hereditary disease in which patients have thousands of colorectal polyps that develop into tumours. Most sporadic colorectal tumours harbour mutations in both *APC* alleles.

**Laboratory of Experimental Oncology, Department of Medical Oncology, University Medical Center, Utrecht, 3584 CG, The Netherlands.*
‡Ludwig Institute for Cancer Research and Department of Cellular and Molecular Medicine, University of California at San Diego, La Jolla, California 92093-0670, USA.
Correspondence to G.J.P.L.K. and D.W.C.
e-mails: g.j.p.l.kops@med.uu.nl; dcleveland@ucsd.edu
doi:10.1038/nrc1714

The mechanisms by which chromosomes are equally separated between two daughter cells have been a challenge to understand ever since Theodor Boveri (in the footsteps of Walther Flemming[1]) showed the dramatic synchronous separation of chromosomes during the first mitotic divisions of fertilized sea urchin eggs over 100 years ago[2]. Boveri further described the detrimental effect of unequal segregation of chromosomes on these cells and their progeny[2]. After von Hansemann had observed many abnormal mitotic figures in samples from various carcinomas in 1890 (REF. 3), it was Boveri, again, who postulated that such misdistribution of chromosomes might be a cause for tumour development and birth defects[4]. Indeed, it now seems that all human aneuploidies (cells that have a chromosome number other than 46) that occur during development result in embryonic lethality — except certain combinations of sex chromosomes and trisomies 13, 18 and 21, which lead to severe birth defects. In addition, most solid tumour cells are aneuploid[5] and various cancer cell lines show 'chromosomal instability' (CIN), meaning that they frequently lose and gain whole chromosomes during divisions[6].

The cause of these observed chromosome imbalances is unknown, but will probably be found in defects in the processes that control chromosome segregation during mitosis (BOX 1). One of these processes is the mitotic checkpoint (also known as the spindle assembly checkpoint) — a complex signalling cascade that is essential for the survival of human cells[7,8]. Evidence indicates that mitotic checkpoint defects contribute to tumorigenesis. However, attacking the machinery that is responsible for chromosome segregation is one of the most successful strategies of clinical chemotherapy. So, gaining a better understanding of mitotic entry, progression and exit is essential, not only for uncovering the causes of CIN, but also for the design of more effective drugs to destroy tumour cells. In this review, we will discuss the mitotic checkpoint as one of the possible causes of CIN in tumour development, and the potential of targeting this checkpoint signalling pathway as a strategy for clinical anticancer therapies.

Aneuploidy and cancer

Since the predictions of Boveri[2,4], it has become clear that most solid tumours are not only aneuploid but have also acquired a number of mutations in oncogenes and tumour-suppressor genes such as *KRAS*, *TP53* (tumour protein p53), *RB1* (retinoblastoma 1), *PTEN* (phosphatase and tensin homologue), *APC* (ADENOMATOUS POLYPOSIS COLI), *BRCA1* (breast cancer 1) and others. This has fuelled the debate over whether aneuploidy is an essential contributor to, or merely a

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Review

Apoptosis, p53, and Tumor Cell Sensitivity to Anticancer Agents[1]

J. Martin Brown[2] and Bradly G. Wouters

Cancer Biology Research Laboratory, Department of Radiation Oncology, Stanford University School of Medicine, Stanford, California 94305

Abstract

A widely held tenet of present day oncology is that tumor cells treated with anticancer agents die from apoptosis, and that cells resistant to apoptosis are resistant to cancer treatment. We suggest, in this review, that this tenet may need to be reexamined for human tumors of nonhematological origin, for two principal reasons: (*a*) cell killing has often been assessed in short term assays that are more influenced by the rate, than the overall level, of cell killing. This has tended to underestimate cell killing for cells not susceptible to apoptosis or having mutant *p53*; and (*b*) conclusions from experiments with normal cells transformed with dominant oncogenes have often been extrapolated to tumor cells. This does not take into account the fact that tumor cells have invariably undergone selection to an apoptotically resistant phenotype. In this review, we examine the impact of these two factors with particular emphasis on the influence of mutations in *p53* on the sensitivity of tumor cells to DNA-damaging agents. We find that because wild-type *p53* predisposes cells to a more rapid rate of cell death after DNA damage, particularly with normal or minimally transformed cells, that short-term assays have led to the conclusion that mutations in *p53* confer resistance to genotoxic agents. On the other hand, if clonogenic survival is used to assess killing in cells derived from actual solid human tumors, then apoptosis and the genes controlling it, such as *p53* and *bcl-2*, appear to play little or no role in the sensitivity of these cells to killing by anticancer drugs and radiation.

Introduction

During the past 10 years, interest of basic scientists and clinicians in the influence of programmed cell death, or apoptosis, on the sensitivity of tumors to anticancer treatment has risen and continues to rise dramatically. A major reason for this interest is that apoptosis is a defined program of cell death that is markedly influenced both positively and negatively by a variety of genes, many of which are mutated and/or dysfunctionally regulated in human cancers (1). Among the most important of these are the tumor suppressor gene *p53* and members of the *bcl-2* gene family (1, 2). The fact that apoptosis is a genetically defined pathway has led to two principal expectations: (*a*) that the genotype of the tumor will be predictive of the outcome of current anticancer therapy; and (*b*) that new therapies based on apoptosis will be superior to present-day anticancer treatments. The requirement for wild-type *p53* for apoptosis after genotoxic damage caused by anticancer agents including irradiation has been well demonstrated, particularly in oncogenically transformed rodent cells and in tissues of lymphoid origin (3, 4). However, the influence of *p53* and other genes on apoptosis in malignant tissues of nonhematological origin is by no means clear. There have also been reports indicating that apoptosis does not correlate with the total cell kill measured by other means following anticancer therapies. In this review, we will focus on tumor cells of nonhematological origin. In particular, we review critically the data underlying the hypothesis that these cancer cells when treated with radiation or chemotherapeutic drugs die of apoptosis, and that cells resistant to apoptosis are resistant to cell kill by anticancer therapy.

Many genes have been identified that affect the extent to which certain cell types undergo apoptosis during normal development and after pathological stress. Together with the assumption that apoptosis plays a major role in cell killing by DNA-damaging agents, these genetic studies have led to the present hypothesis that tumors with mutations in *p53*, high levels of bcl-2, or high ratios of bcl-2:bax should be resistant to cancer treatment (1, 2, 5). Because there is now a wealth of data from clinical studies in which outcome has been correlated with the status of these and other genes affecting apoptosis, this hypothesis would seem an easy one to test. However, a major problem with such analyses is that it is often impossible to separate treatment sensitivity from patient prognosis. For example, tumors with mutated *p53* can be more anaplastic, can have a higher proportion of proliferating cells, can be more metastatic, and in general can have a more aggressive phenotype that similar tumors with wild-type *p53* (6). This can lead to a worse prognosis for patients whose tumors have mutated *p53* independent of treatment sensitivity (7). Having said this, there are numerous examples in the literature where *p53* mutations (or high levels of p53 protein by immunohistochemistry) either do not affect patient prognosis (8, 9) or lead to better outcome after treatment (10, 11). In a comprehensive review of the clinical significance of *p53* mutations in human tumors, Bosari and Viale (12) concluded (in 1995) that a definite answer could not yet be given to the question or whether *p53* aberrations led to a more aggressive phenotype or to treatment resistance.

Apoptosis and Sensitivity to Anticancer Therapy: The Present View. As we point out above, because mutations in *p53* or other genes may affect tumor aggressiveness and patient prognosis, it is difficult to obtain from clinical data an answer to the question of the role of *p53* or of apoptosis in treatment sensitivity. However, experimental systems can be not only free of such biases, they can also use modern gene knockout, transgene, and other molecular techniques to answer the narrower question of: "Does the level of apoptosis and/or genes controlling apoptosis affect the sensitivity of cancer cells to killing by genotoxic agents?"

The present view is that this is the case (1, 2, 5, 13, 14). It has become widely accepted that cell death after DNA damage by anticancer agents is primarily the result of apoptosis, and that cells that can evade apoptosis will be resistant to cell killing. Often cited for this view, and in particular the role of mutated *p53* in radiation and anticancer drug resistance, are pioneering studies with dominant oncogene-transformed normal fibroblasts from embryos of *p53* wild-type ($p53^{+/+}$) and *p53* knockout mice ($p53^{-/-}$) (15, 16), as well as highly significant associations of mutated *p53* with drug resistance in the National Cancer Institute panel of 60 cell lines used for screening novel potential anticancer drugs (17).

However, despite the seemingly strong case that cells die from cancer treatment due to apoptosis largely controlled by wild-type p53,

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[2] To whom requests for reprints should be addressed, Cancer Biology Research Laboratory, GK103, Department of Radiation Oncology, Stanford University School of Medicine, Stanford, CA 94305-5468.

CHECKING ON DNA DAMAGE IN S PHASE

Jiri Bartek, Claudia Lukas and Jiri Lukas

Abstract | The precise replication of the genome and the continuous surveillance of its integrity are essential for survival and the avoidance of various diseases. Cells respond to DNA damage by activating a complex network of the so-called checkpoint pathways to delay their cell-cycle progression and repair the defects. In this review we integrate findings on the emerging mechanisms of activation, the signalling pathways and the spatio-temporal organization of the intra-S-phase DNA-damage checkpoint and its impact on the cell-cycle machinery, and discuss its biological significance.

INTRA-S-PHASE CHECKPOINT
Replication-independent mechanism that reduces the rate of DNA synthesis in response to DNA double-strand breaks.

Danish Cancer Society, Institute of Cancer Biology, Strandboulevarden 49, DK-2100 Copenhagen, Denmark.
Correspondence to J.B.
e-mail: bartek@biobase.dk
doi:10.1038/nrm1493

During the DNA-synthesis (S) phase of each cell-division cycle that is undertaken by any of the 10^{13}–10^{14} cells in our body, one of the miracles of life occurs — the entire human genome, which is composed of some three billion base pairs, is faithfully replicated. A high fidelity of duplication and the overall maintenance and error-free transmission of the genetic material to subsequent generations are critical to survival at both the cellular and organismal levels. Preserving the integrity of the genome is even more demanding, however, given the continuous threat of adverse genetic changes from a plethora of DNA lesions. These can be caused by environmental or endogenous genotoxic insults such as ionizing or ultraviolet radiation (IR or UV, respectively), various chemicals and drugs, and reactive cellular metabolites. Consequently, continuous surveillance of the genetic material and prompt action to repair any DNA damage, or to eliminate hazardous, genetically unstable cells, are required. To cope with this challenging task, all eukaryotes have evolved an elaborate network of molecular mechanisms to detect unreplicated or aberrant DNA structures, to spread the alert signal, and to respond through the coordinated activities of diverse DNA-repair and so-called cell-cycle-checkpoint pathways[1–3].

Checkpoints were traditionally defined as molecular signalling cascades that promote cell-cycle delay or arrest in response to DNA damage, thereby providing more time for the repair of the damage[4]. Recent evidence also points to further, mutually integrated roles of the checkpoint machinery in the activation of DNA repair, chromatin remodelling, modulation of transcriptional programmes and the optional triggering of permanent cell-cycle withdrawal (a process that is known as cellular senescence) or cell death[1,5–7].

Diverse and partly overlapping or redundant checkpoint pathways operate in various cell-cycle phases and, to a more limited extent, also in quiescent and even terminally differentiated cells[2,8,9]. Proliferating cells that traverse through G1 or G2 phases can respond to genotoxic stress by activating checkpoints that impose shorter or durable cell-cycle arrests in G1 or G2, before re-entry into S phase or mitosis (M phase), respectively. By contrast, cells that experience genotoxic stress during DNA replication only delay their progression through S phase in a transient manner, and if damage is not repaired during this delay they exit S phase and arrest later when reaching the G2 checkpoint.

Possibly reflecting the lack of a prominent intra-S-phase cell-cycle arrest, the mammalian S-phase checkpoints have often been thought to have only a minor role compared with the more robust G1 and G2 checkpoints, which have attracted more attention from cell and cancer biologists. By focusing on the S-phase checkpoints — particularly the replication-independent INTRA-S-PHASE CHECKPOINT response to DNA double-strand breaks (DSBs) — we hope to remedy

APOPTOSIS AND GENOMIC INSTABILITY

Boris Zhivotovsky and Guido Kroemer‡*

Abstract | Genomic instability is intrinsically linked to significant alterations in apoptosis control. Chromosomal and microsatellite instability can cause the inactivation of pro-apoptotic pathways. In addition, the inhibition of apoptosis itself can be permissive for the survival and ongoing division of cells that have failed to repair DNA double-strand breaks, experience telomere dysfunction or are in an abnormal polyploid state. Furthermore, DNA-repair proteins can regulate apoptosis. So, genomic instability and apoptosis are intimately linked phenomena, with important implications for the pathophysiology of cancer.

GENOMIC INSTABILITY
The failure to transmit an accurate copy of the entire genome from one cell to its two daughter cells. Note that this term does not describe a state but, rather, a process.

CENTROSOME
A specialized organelle that duplicates during interphase and that constitutes the centre of the mitotic spindle.

**Institute of Environmental Medicine, Karolinska Institutet, Box 210, Nobels väg 13, SE-171 77 Stockholm, Sweden.*
‡Centre National de la Recherche Scientifique-UMR8125, Institut Gustave Roussy, 38 rue Camille Desmoulins, F-94805 Villejuif, France.
e-mails: kroemer@igr.fr; boris.zhivotovsky@imm.ki.se
doi:10.1038/nrm1443

Genome integrity and cell proliferation and survival are regulated by an intricate network of pathways that include cell-cycle checkpoints, DNA repair and recombination, and programmed cell death. Permanent or transient GENOMIC INSTABILITY (BOX 1), which represents one of the fundamental characteristics of cancer, might be ascribed to deficiencies in numerous cellular processes including mitotic-checkpoint regulation, and DNA-damage signalling and repair, as well as telomere maintenance and CENTROSOME function. This review will focus on the complex interplay between genomic (in)stability and apoptosis regulation (BOX 2) that participates in carcinogenesis.

The relationship between genomic integrity and cell-death regulation can follow at least three different non-exclusive patterns, all of which might be important for the development of cancer. First, genomic instability can lead to the mutation, or altered expression levels, of cell-death regulators. Second, disabled apoptosis can favour genomic instability. Indeed, numerous cellular mechanisms enforce the rule 'better dead than wrong', which means that cells that have a damaged genome or are afflicted by many disorders will be aborted by apoptosis — thereby avoiding the propagation of potentially oncogenic mutations. DNA double-strand breaks (DSBs), telomere dysfunction, illicit POLYPLOIDY or abnormal mitoses can directly trigger apoptosis through a default pathway. However, if apoptosis is inhibited for some reason, this increases the risk of CHROMOSOMAL INSTABILITY (CIN) at several levels, and cells that are sufficiently fit to survive can be at a growth advantage, which can lead to cancer. Third, a single protein or process might be involved in the control of both apoptosis and genomic instability, which allows 'crosstalk' between the processes.

Here, we will discuss these different possibilities, their molecular mechanisms and their possible impact on carcinogenesis.

Genomic instability: disabling apoptosis

Genomic instability is a hallmark of cancer, the pathogenesis of which is also characterized by specific genetic and epigenetic changes that can result in defective apoptosis[1]. It is tempting to assume that genetic instability, after selection, will result in the expansion of a cell population that is relatively resistant to apoptosis induction. Because disabling apoptosis, in itself, might favour genetic instability (see below), it becomes plausible that both mechanisms might cooperate to increase the oncogenic and metastatic potential of transformed cells. During the initial stages of oncogenesis, a series of random alterations in the unstable genome can lead to a collection of nonrandom genetic alterations that affect a restricted set of oncogenes (for example, oncogenes that encode apoptosis inhibitors) and tumour-suppressor genes (which might encode apoptosis facilitators). These genetic alterations would be nonrandom,

Keren *et al.* increase the complexity of such devices by using yet another biomolecule, the RecA protein, which plays a major role in genetic recombination of the cell. Multiple RecA proteins polymerize at a single-stranded DNA (ssDNA) molecule. By homologous recombination, the resulting DNA-protein complex recognizes and binds to the complementary target sequence present in double-stranded DNA (dsDNA). Keren *et al.* treat the DNA-protein complex with silver ions, which are reduced by aldehyde groups previously generated in the dsDNA target. The resulting small silver grains are later used for the wet-chemical deposition of gold.

This procedure leads to the formation of a conductive wire, with an insulating gap precisely at the position where RecA was bound (see panel A in the figure). The information encoded in the DNA molecules thus replaces the masks used in conventional lithography, while the RecA protein serves as the resist. This approach should, in principle, work with high resolution over a broad range of length scales from nanometers to many micrometers.

Molecular lithography also enables the generation of branchpoints (three-way junctions) in linear DNA fragments. This is accomplished with the use of a dsDNA fragment containing a single-stranded end in the initial RecA polymerization step (see panel B). Treatment of the DNA-protein complex with polymerase leads to the sequence-specific formation of a stable three-way junction, which can later be used for the fabrication of more complex components, such as three-terminal electronic devices. This clear demonstration of the feasibility of biomolecular construction is likely to trigger further efforts to design advanced host systems required for efficient in situ generation of nanostructured DNA networks, and for their site-specific attachment and connection with the macroscopic world.

Molecular lithography can also be used for the sequence-specific positioning of molecular objects (see panel C). To this end, the ssDNA used for polymerization of the RecA monomers is modified with molecular entities that are functional devices or allow for the binding of such devices. To illustrate this possibility, Keren *et al.* introduced biotin groups in the ssDNA, enabling the specific docking of streptavidin-coated gold nanoparticles that were later used for the growth of a metal island. This feature of molecular lithography should stimulate extensive follow-ups, taking advantage of previous work on the DNA-directed assembly of metal and semiconductor nanoparticles (*7*) and/or proteins (*8*).

Additional perspectives for molecular lithography may be opened by incorporating novel proteins obtained by molecular biotechnology techniques, such as directed evolution and phage-display. These approaches have already allowed the in vitro production of protein linker units that recognize semiconductor quantum dot surfaces (*9*). The linkers can thus be used to assemble individual nanometer-size inorganic particles into two- and three-dimensional superstructures.

The combination of molecular lithography and protein bioengineering should open up ways to generate and integrate supramolecular nanoparticle networks and microelectronic devices with biomaterials that possess distinct functionality with respect to enzymatic activity and redox properties (*10*). The highly evolved catalytic turnover of many enzymes and the mechanical or electronic transduction properties of, for example, motor proteins, oxidoreductases, and ion channels, should enable new applications in the areas of sensing, catalysis, and electronics. The joint venture of biotechnology and electronic engineering promises plenty of excitement from future developments.

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PERSPECTIVES: CANCER

Addiction to Oncogenes—the Achilles Heal of Cancer

I. Bernard Weinstein

A single cancer cell frequently contains mutations in multiple genes, gross chromosomal abnormalities, and widespread changes in its gene expression profile. An axiom in cancer research is that the multistage process of tumor formation (*1*) is driven by progressive acquisition of activating mutations in dominant growth-enhancing genes (oncogenes) and inactivating mutations in recessive growth-inhibitory genes (tumor suppressor genes) (*2*). Epigenetic (nonmutational) abnormalities leading to increased or decreased expression of these genes, respectively, are also important for tumorigenesis (*2*–*4*). Since the discovery of oncogenes about 20 years ago, more than 100 oncogenes and at least 15 tumor suppressor genes have been identified, and the list keeps growing. Oncogenes and tumor suppressor genes are important not only for cell proliferation but also for cell fate determination (differentiation, senescence, and apoptosis), their effects often depending on the type of cell in which they are expressed. Thus, overexpression of a given oncogene can enhance growth in one cell type but inhibit growth or induce apoptosis in another (*2*–*4*).

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The author is at the Herbert Irving Comprehensive Cancer Center, Columbia University, New York, NY 10032, USA. E-mail: weinstei@cancercenter.columbia.edu

A tantalizing question still under debate is whether an oncogene that is crucial for the initial development of a specific tumor is required for maintaining the malignant phenotype of that tumor. The study by Jain *et al*. on page 102 of this issue (*5*) addresses this question. By unraveling the molecular circuitry that maintains the biologic properties of cancer cells, we will be better able to predict selective molecular targets for cancer therapy. Jain and colleagues (*5*) engineered a conditional transgenic mouse to overexpress the *myc* oncogene, which induced formation of highly malignant osteogenic sarcoma. They discovered that brief loss of *myc* overexpression caused the tumor cells to differentiate into mature osteocytes that formed histologically normal bone. It is also intriguing that subsequent reactivation of *myc*, rather than restoring tumor growth as would be predicted, instead induced apoptosis of the tumor cells.

These findings are consistent with other data showing that cancer cells are often "addicted to" (that is, physiologically dependent on) the continued activity of specific activated or overexpressed oncogenes for maintenance of their malignant phenotype. For example, Felsher and Bishop (*6*) showed that transgenic mice expressing the *myc* oncogene in hematopoietic cells developed malignant T cell leukemias and acute myeloid leukemias. However, when this gene was switched off the leukemic cells underwent proliferative arrest, differentiation, and apoptosis. Pelengaris *et al*. (*7*) targeted expression of an activatable

TUMOUR BIOLOGY

Senescence in premalignant tumours

Oncogene-induced senescence is a cellular response that may be crucial for protection against cancer development[1,2], but its investigation has so far been restricted to cultured cells that have been manipulated to overexpress an oncogene. Here we analyse tumours initiated by an endogenous oncogene, *ras*, and show that senescent cells exist in premalignant tumours but not in malignant ones. Senescence is therefore a defining feature of premalignant tumours that could prove valuable in the diagnosis and prognosis of cancer.

We used a mouse model for cancer initiation in humans: the animals have a conditional oncogenic K-*rasV12* allele that is activated only by the enzyme Cre recombinase[3], causing them to develop multiple lung adenomas (premalignant tumours) and a few lung adenocarcinomas (malignant tumours). Senescence markers previously identified in cultured cells were used to detect oncogene-induced senescence in lung sections from control mice (expressing Cre) and from K-*rasV12*-expressing mice (expressing Cre and activated K-*rasV12*). We analysed $p16^{INK4a}$, an effector of *in vitro* oncogene-induced senescence[1], and *de novo* markers that we identified by using DNA microarray analysis of *in vitro* oncogene-induced senescence (see supplementary information). These *de novo* markers are $p15^{INK4b}$ (also known as CDKN2B), Dec1 (BHLHB2) and DcR2 (TNFRSF10D). In addition, we looked for two features evident in cultured senescent cells, namely the expression of senescence-associated β-galactosidase[4] and the presence of senescence-associated heterochromatin foci[5].

Staining with antibodies against $p16^{INK4a}$, $p15^{INK4b}$, Dec1 and DcR2 revealed abundant positive cells in adenomas, whereas adenocarcinomas were essentially negative (Fig. 1a). By contrast, the proliferation marker Ki-67 revealed a weak proliferative index in adenomas compared with adenocarcinomas (Fig. 1a). Lung cryosections from K-*rasV12* mice stained for senescence-associated β-galactosidase gave an intense signal in the adenomas, whereas adenocarcinomas gave a weak or negative signal (Fig. 1b). Adenomas were also strongly positive for HP1-γ, which indicates the formation of senescence-associated heterochromatin foci, whereas adenocarcinomas were negative (Fig. 1c). These results were consistently found when using K-*rasV12* mice carrying Cre transgenic alleles that were expressed either inducibly by tamoxifen (Cre-ER) or constitutively (CMV-Cre)[3]; 5–10 mice were analysed for each marker. The results have also been confirmed by immunoblotting and by quantitative real-time polymerase chain reaction with reverse transcription (see supplementary information; these analyses also included $p19^{ARF}$, an effector of senescence-associated β-galactosidase[6]).

Extending these observations, we combined the K-*rasV12* allele with a transgenic Cre allele that targets the expression of the oncogene to the pancreas. These compound mice develop premalignant lesions (pancreatic intraductal neoplasias) that progress into malignant ductal adenocarcinomas (C.G., A.J.S. and M. Barbacid, unpublished results). As in lung adenomas, these premalignant lesions were positive for our oncogene-induced senescence markers, whereas ductal adenocarcinomas were negative. Similarly, chemically induced skin papillomas, which harbour H-*ras* oncogenic mutations, were also positive for oncogene-induced senescence markers (see supplementary information).

We infer from our findings that oncogene-induced senescence may help to restrict tumour progression. In most cells, oncogenic K-*ras* signalling is attenuated and is therefore not sufficient to trigger tumour formation or senescence[3,7]. Presumably, rare cells that do not fully attenuate oncogenic Ras are at the origin of both premalignant and malignant tumours. We conclude that a substantial number of cells in premalignant tumours undergo oncogene-induced senescence, but that cells in malignant tumours are unable to do this owing to the loss of oncogene-induced senescence effectors such as $p16^{INK4a}$ or p53.

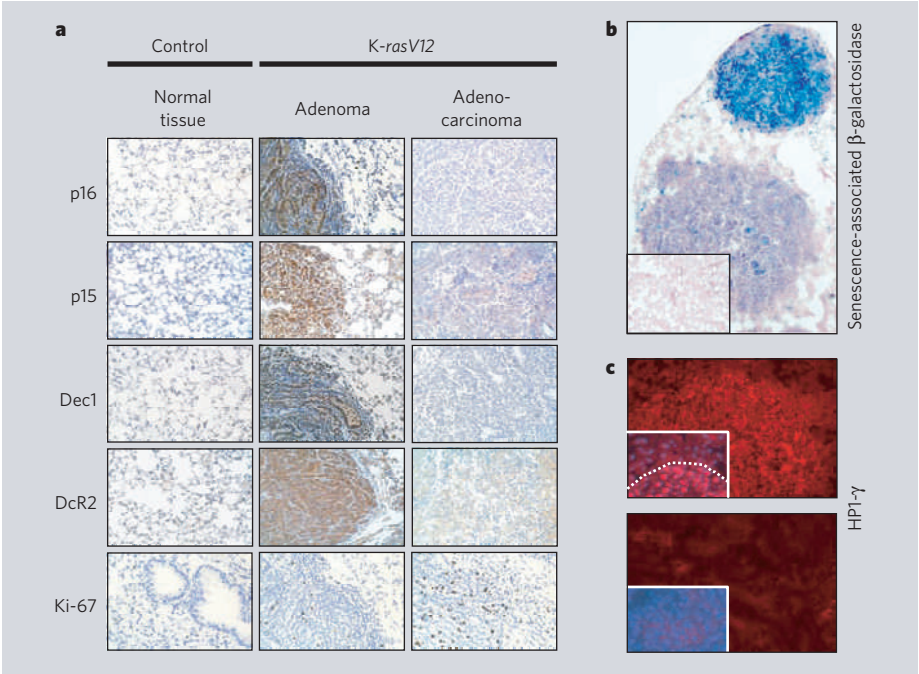

Figure 1 | Premalignant lung adenomas induced by oncogenic K-*ras* are positive for markers of senescence, whereas malignant adenocarcinomas are negative. **a,** Immunohistochemical analysis of the different tissue types for the indicated proteins. **b,** Senescence-associated β-galactosidase expression. A lung section is shown that contains one adenoma (top, blue stained) and one adenocarcinoma (bottom). Inset, negative control. **c,** Immunofluorescence using anti-HP1-γ staining of adenoma (top) and adenocarcinoma (bottom). Insets, double staining with anti-HP1-γ (red) and DAPI (4,6-diamino-2-phenylindole, which stains cell nuclei) (blue). Dotted line, boundary between adenoma and normal tissue. (See supplementary information for further details.)

Manuel Collado*, Jesús Gil†, Alejo Efeyan*, Carmen Guerra*, Alberto J. Schuhmacher*, Marta Barradas*, Alberto Benguría‡, Angel Zaballos‡, Juana M. Flores§, Mariano Barbacid*, David Beach||, Manuel Serrano*

*Spanish National Cancer Centre (CNIO), Madrid 28029, Spain
e-mail: mserrano@cnio.es
†MRC Clinical Sciences Centre, Imperial College, Hammersmith, London W12 0NN, UK
‡Spanish National Centre of Biotechnology (CNB-CSIC), Madrid 28049, Spain
§Department of Animal Surgery and Medicine, Complutense University, Madrid 28040, Spain
||Centre for Cutaneous Research, Institute of Cell and Molecular Science, London E1 2AT, UK

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bird and land-cover databases would surely yield quantitative evidence of the influence of habitat. Practically all ecologists agree that species have habitat requirements that limit where they can live — tropical trees cannot survive on the Arctic tundra. Graves and Rahbek are correct that neutral theory cannot predict the resulting influence of habitat on community composition because it ignores species differences entirely.

But does the importance of habitat disagree with the letter or the intent of neutral theory? In other words, does it contradict the overall principle of dispersal-assembly?

Not necessarily. The idea of dispersal-assembly is not that differences between species do not exist — they are the inevitable result of disparate evolutionary histories. Rather, the idea is that species similarities, not their differences, lead them to find the same region habitable and to coexist. Neutral theory applies only in that realm of intermingling, where species are similar.

Habitat influence on species' distributions at any scale does indicate a role for niche-assembly, which has implications for ecological dynamics. The species that differ in the habitat they do best in cannot out-compete each other. Their differences allow them to coexist stably in the landscape.

However, unless habitat and species change in lock-step, habitat effects do not rule out a simultaneous role for dispersal-assembly. As Graves and Rahbek acknowledge, their observations limit only the spatial scale and groups of species within which neutral theory's unstable ecological dynamics may apply. Furthermore, differences between species in habitat requirements can arise from sources that are consistent with dispersal-assembly in a heterogeneous landscape over evolutionary timescales, such as from local selection for capabilities on a par with those of competitors. Selection for the avoidance of competition (or niche-assembly) may not be the evolutionary origin of these differences.

More empirical work is needed to distinguish between niche-assembly and dispersal-assembly on both ecological and evolutionary timescales. We also need to understand the implications of this distinction, and more refined ones, for judging the robustness and resilience of communities in the face of anthropogenic change. ■

Annette Ostling is in the Department of Ecology and Evolutionary Biology, Guyot Hall, Princeton University, Princeton, New Jersey 08544-1003, USA.
e-mail: aostling@princeton.edu

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CANCER

Crime and punishment

Norman E. Sharpless and Ronald A. DePinho

Cellular senescence stops the growth of cells. This process, first glimpsed in cell culture, is now confirmed by *in vivo* evidence as a vital mechanism that constrains the malignant progression of many tumours.

Societies have traditionally taken three approaches to handling recidivist criminals: exile, execution and lifetime imprisonment. It seems that human cells use similar strategies to prevent rogue cells harbouring dangerous mutations from turning into fully fledged cancers. Epithelial tissue, such as that lining the airways and intestines, continuously renews and sloughs off, thereby sentencing some precancerous cells to extra-corporeal exile. There is also a cellular version of the death penalty — apoptosis, a well-established anticancer mechanism. And in this issue, four groups[1–4] report striking *in vivo* evidence that the body can subject potential cancer cells to the equivalent of a life-sentence: cellular senescence.

Senescence is a specific form of stable growth arrest provoked by diverse stresses, including the enforced expression of cancer-promoting genes in cultured cells. This 'oncogene-induced senescence' (OIS)[5] is linked to known cancer pathways in cultured cells, notably the ARF–p53 and $p16^{INK4a}$–RB pathways (Fig. 1). But whether OIS is an authentic anticancer process *in vivo*, or simply an artefact of enforced oncogene expression in cells experiencing culture shock[6], has been controversial.

This issue is settled by the new papers[1–4], which show that OIS occurs *in vivo* in several diverse precancerous tissues from both human and mouse. In addition, the work identifies much-needed markers of senescence, and further delineates the molecular underpinnings of this key tumour-suppressing process. A compelling feature of these studies is the consistency of OIS in response to a variety of cancer-causing mutations in different human tumour types and mouse-model systems. At the same time, the reports reveal that the molecular circuitry of OIS may be wired differently among tumour types.

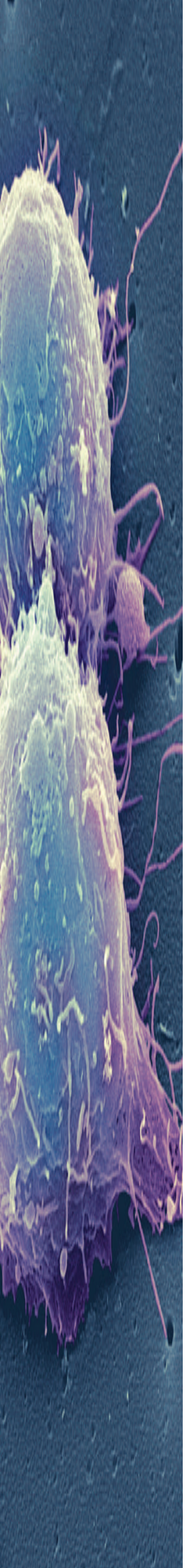
Michaloglou *et al.* (page 720)[1] worked with cultures of human melanocytes (pigmented skin cells) and nevi (skin moles, the benign precursors of malignant melanoma). They found that nevi harbouring mutations of the BRAF protein (mutations that are frequently found in melanomas) have robust expression of senescence markers and do not seem to proliferate. In melanoma cells, however, senescence is extinguished and proliferation accelerated.

Curiously, the tumour suppressor $p16^{INK4a}$ — a known activator of senescence that is deleted in melanoma cells — showed spotty expression in nevi, and experimental depletion of $p16^{INK4a}$ failed to increase BRAF-induced senescence in melanocyte cultures. Mutated BRAF in melanocytes also failed to induce the ARF and p53 tumour suppressors, two proteins integral to the activation of senescence in many systems. These results expose serious gaps in our understanding of the genes and pathways that function to constrain the transformation of nevi into lethal melanomas.

Exploring the evolution of prostate cancer, Chen *et al.* (page 725)[2] discovered senescence in early-stage prostate abnormalities in humans and in mice engineered to sustain prostate-specific deletion of the PTEN tumour-suppressor gene. However, in contrast to the situation in melanocytes, prostate OIS is dependent on p53, and co-deletion of PTEN and p53 cancelled senescence, promoting full-blown prostate cancer. Parallel studies using mouse models to dissect the role of the *Ras* oncogene in the lung and pancreas[3] and in lymphoid cells[4] reinforced similar principles. So, although previous work has established that the role of p53 as a tumour suppressor depends on its ability to mediate apoptosis, these papers emphasize that p53 can also mediate senescence in primary tumours.

Collado *et al.* (page 642)[3] address a crucial need for better *in vivo* markers of OIS. So far, the gold standard has been the detection of an enzymatic activity associated with senescence (that of SA-β-gal)[7]. Although SA-β-gal has been used successfully to analyse human and mouse samples, this marker is not molecularly well-defined and demonstrates background activity in certain organs. Collado *et al.* employed an ingenious microarray screen to identify a small set of genes, the expression of which correlates strongly with senescence induced by the ERK protein. (ERK mediates the effects of certain cancer-causing mutations.) The correlations with gene expression are not seen when ERK is induced in the absence of senescence. These markers of OIS include protein-encoding genes and at least three RNA-encoding genes that are relevant to mouse tumour models of different tissues. These markers might predict OIS in precancerous abnormalities in humans.

Braig *et al.* (page 660)[4] provide a penetrating biochemical view of senescence. Their experiments were guided by the observation of unusual foci of tightly packed DNA in senescent cells[8]. These foci possessed features of a form of silenced DNA called heterochromatin,



Intrinsic tumour suppression

Scott W. Lowe[1], Enrique Cepero[1] & Gerard Evan[2]

[1]*Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, New York 11724, USA*
[2]*Cancer Research Institute, University of California at San Francisco, San Francisco, California 94143, USA (e-mail: lowe@cshl.edu)*

Mutations that drive uncontrolled cell-cycle progression are requisite events in tumorigenesis. But evolution has installed in the proliferative programmes of mammalian cells a variety of innate tumour-suppressive mechanisms that trigger apoptosis or senescence, should proliferation become aberrant. These contingent processes rely on a series of sensors and transducers that act in a coordinated network to target the machinery responsible for apoptosis and cell-cycle arrest at different points. Although oncogenic mutations that disable such networks can have profound and varied effects on tumour evolution, they may leave intact latent tumour-suppressive potential that can be harnessed therapeutically.

Cancers arise by an evolutionary process as somatic cells mutate and escape the restraints that normally rein in their untoward expansion. Suppressing the emergence of such autonomous cells is an evolutionary imperative of metazoans, particularly in large, long-lived organisms where cells in regenerative tissues retain the potential for neoplastic havoc throughout life. Consequently, multiple mechanisms have arisen to forestall uncontrolled cell division. Some of these are devices within the cell, such as those that limit cell-cycle progression, whereas others are social signals that prompt a cell to remain within its supportive microenvironment. In combination, these tumour-suppressing mechanisms are remarkably effective; on average, cancers arise less than once in a human lifetime, despite trillions of potential target cells, each harbouring hundreds of susceptible cancer-causing genes, all subject to a significant mutation rate. Yet more remarkable is the fact that our tumour-defence systems can discriminate between neoplastic (abnormally growing) and normal cellular states and efficiently quell the former without suppressing the latter.

Insight into the mechanisms that constrain neoplastic progression has come from the realization that many, perhaps all, networks that drive cell proliferation harbour intrinsic growth-suppressive properties. Such innate inhibitory functions obscure any immediate selective advantage that mutations in such pathways might otherwise confer. Because no single pathway confers a net growth advantage, any proto-cancer cell acquiring any single oncogenic mutation is effectively trapped in an evolutionary cul-de-sac. By contrast in normal cells, coordinated extracellular cues activate multiple pathways in concert. In this way, the inherent growth-suppressive activity of each pathway is gated by another, thereby unlocking the cell's proliferative potential (Fig. 1). The nature of the coupling of growth-inhibitory programmes to proliferative networks, and its implications for understanding the evolution and treatment of cancers, are the focus of this review.

Oncogene-induced apoptosis

Cell proliferation and cell death are such diametrically opposed cellular fates that the discovery that the two are linked and interdependent processes was a great surprise[1,2]. There is little mechanistic overlap between the machineries driving proliferation and apoptosis. Rather, the two processes are coupled at various levels through the individual molecular players responsible for orchestrating cell expansion. Importantly, the same players are often targets for oncogenic mutations, and in many instances, mutations that drive proliferation cooperate with those that uncouple proliferation from apoptosis during transformation and tumorigenesis[2,3]. But, although the phenomenon of oncogene-induced apoptosis is now generally accepted as an innate tumour-suppressive mechanism, we have only recently begun to glimpse the diversity and complexity of mechanisms by which oncogenic lesions engage the cell suicide machinery.

At least two distinct general programmes trigger apoptosis, each regulated at many levels (Fig. 2). The 'intrinsic' pathway is the primary death programme responsive to the signals of survival factors, cell stress and injury[4–6]. The central conduit of this pathway is the mitochondrion, the intermembrane space of which sequesters a variety of pro-apoptotic effectors that, when released, trigger cellular demise. Mitochondrial permeability is, in turn, determined by the balance between the pro-apoptotic Bax/Bak proteins and their anti-apoptotic Bcl2/BclXL cousins. The activity of these proteins are positively or negatively regulated by the various BH3-only members (Bcl2 family members that contain a single Bcl2 homology-3 domain), each acting as the terminal effector of distinct signalling pathways. According to this simple model, apoptosis occurs when the protective Bcl2/BclXL buffer is breached by the sum of all the active BH3-only proteins, resulting in the net dominance of the pro-apoptotic Bax/Bak proteins, which then permeabilize the mitochondria to release pro-apoptotic factors. One such factor, cytochrome *c*, acts together with

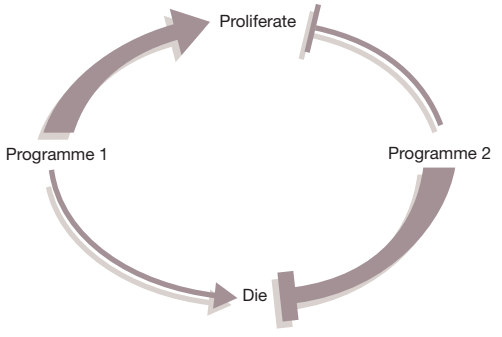
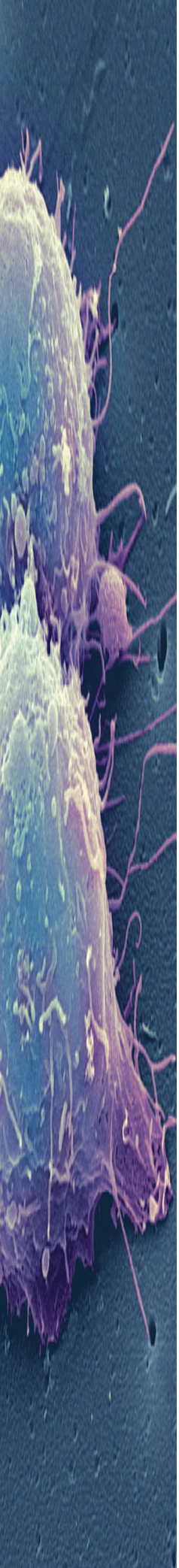


Figure 1 Example of an obligate combinatorial signalling network. Programme 1 drives proliferation and apoptosis, and Programme 2 blocks both. For each cell fate, dominant components are shown as thick lines. Concerted activation of both programmes together leads to cell expansion because Programme 1 overcomes the growth inhibition of Programme 2, and Programme 2 overcomes the lethality of Programme 1. However, activation of either programme on its own triggers cell-death (Programme 1) or senescence (Programme 2).



G1 cell-cycle control and cancer

Joan Massagué

Cancer Biology and Genetics Program, and Howard Hughes Medical Institute, Box 116, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York 10021, USA (e-mail: j-massague@ski.mskcc.org)

Before replicating DNA during their reproductive cycle, our cells enter a phase called G1 during which they interpret a flood of signals that influence cell division and cell fate. Mistakes in this process lead to cancer. An increasingly complex and coherent view of G1 signalling networks, which coordinate cell growth, proliferation, stress management and survival, is helping to define the roots of malignancies and shows promise for the development of better cancer therapies.

The past three decades have seen an unrelenting quest to understand the reproduction of normal and cancerous cells. Cell reproduction entails replication of the DNA followed by division of the nucleus and partitioning of the cytoplasm to yield two daughter cells. This sequential routine is known as the 'cell cycle' and, as a problem that has fascinated biologists since the mid-nineteenth century, we are only just beginning to understand how it works.

Cells in early embryos can proceed through continuous cycles of DNA replication and nuclear division at astonishing speed. DNA replication starts as soon as mitosis ends and a full cycle of cell division is completed in a mere half hour. But as embryogenesis unfolds and the demands of cell life in a complex environment set in, a bureaucracy arises. A gap called G1 phase is incorporated between nuclear division (M phase) and DNA synthesis (S phase); another gap called G2 phase occurs between S and M (Fig. 1b). These gaps allow for the repair of DNA damage and replication errors. But above all, G1 is a period when many signals intervene to influence cell division and the deployment of a cell's developmental programme. Diverse metabolic, stress and environmental cues are integrated and interpreted during this period. On the basis of these inputs, the cell decides whether to enter S phase or pause. Moreover, in multicellular organisms the behaviour of a cell must obey dictums from its neighbours. To this end, during G1 the cell makes further decisions regarding whether to self-renew, differentiate or die.

The proper interpretation and execution of these inputs is a delicate business and, not surprisingly, mistakes lead to cancer. When mutations allow a cell to remain in a proliferative state and avoid terminal differentiation and death, that cell is poised to escalate in its degenerate behaviour. It can acquire additional mutations for invasion of surrounding tissues and metastatic re-creation of the tumour at distant organs. Many oncogenes and tumour suppressor genes, as well as the therapies that target them, can be linked to faulty G1 control.

How then is the decision to initiate DNA replication controlled in our cells at the molecular level? Ongoing work is providing an ever more complex but coherent answer to this question. Here, I discuss examples of signal transduction pathways that influence G1 progression, I describe how these processes affect cancer formation, and finally, I consider current ideas on potential therapeutic interventions. The recent progress in these areas is so extensive that this review can only distill key principles with little room for personal opinion or comprehensive detail.

A G1 engine of cyclins and kinases

Compared to DNA replication and mitosis, which follow canonical steps that vary little from cell to cell, the steps controlling entry and progression through G1 are largely dependent on cell type and context. A stem cell that is constantly replenishing the intestinal lining, a lymphocyte suddenly stimulated by antigen, or an angioblast responding to vascular injury, all proceed through G1 phase under different circumstances, different signals, different developmental programmes and with different risks of malignant transformation. Ultimately, however, to enter S phase all cells must fulfill the same essential requirement: they must activate cyclin-dependent kinases (CDKs).

CDKs are protein kinases that require binding to a cyclin subunit to become catalytically competent[1,2]. Different members of the CDK family, in association with different cyclins, turn key switches throughout the cell cycle; other family members regulate transcription, differentiation, nutrient uptake and other functions. Cyclin–CDK complexes are regulated by phosphorylation and protein interaction events that tightly control the timing and extent of CDK activation. The prototypic CDK, Cdk1, associates with cyclins A and B, and acts at the G2/M interface (Fig. 1). The progressive accumulation of A and B cyclins during the cell cycle and their abrupt degradation at the onset of anaphase, mediates entry and exit from mitosis, respectively. The drop in Cdk1 activity at the end of M phase allows DNA chromosomal sites known as replication origins to be loaded with a pre-replicative complex (PRC) (refs 3, 4; Fig. 2). This complex contains ORC (origin of replication complex), the kinase Cdc6/18 and Ctd1 (Cdc10-dependent transcript 1), and loads MCM (mini-chromosome maintenance) proteins onto the DNA, licensing these sites for the initiation of replication.

G1 CDKs trigger DNA replication. In higher eukaryotes the G1 CDKs include Cdk2, which combines with E-type cyclins (E1, E2) and cyclin A (refs 1, 2). On Cdk2 activation, PRCs recruit DNA helicases, primases and polymerases, causing unwinding of the double helix and DNA replication[3,4] (Fig. 2). Cdk activity is essential for the unwinding step, and several components of the PRC become phosphorylated in the process. The newly replicated origins cannot reassemble new PRCs until CDK activity once again drops at the end of mitosis. Mitosis in turn will not proceed until DNA replication is completed. Together, these events ensure that DNA will be replicated once and only once, per cell cycle[3,4]. The identities of the CDK substrates that directly trigger DNA replication remain unknown and a stinging reminder of how much we still do not know about how cell reproduction works.

The scheme summarized here is well supported by experimental evidence, but it cannot be taken too rigidly. For example, E-type cyclins are largely dispensable for mouse development (although cells lacking cyclin E have problems loading MCM onto DNA)[5]. Even more strikingly,

ELSEVIER

Hitting their targets: an emerging picture of E2F and cell cycle control

Alexandre Blais and Brian David Dynlacht

Understanding the role of transcription factors in governing cell-cycle progression in mammalian cells has been hindered until recently by a relative lack of genetic and genomic approaches. New approaches that harness the power of ChIP and combine this technique with DNA microarrays and bioinformatics have identified direct, physiological targets and have significantly altered our view of the E2F transcription factor that is known to play a role in regulation of cell-cycle progression. Further, the identification of additional E2F family members and factors that function in concert with E2F have considerably expanded our picture of the genetic programs that are governed by this essential regulatory factor.

Addresses
Department of Pathology, MSB 504, New York University School of Medicine and NYU Cancer Institute, 550 First Avenue, New York, New York 10016, USA
e-mail: brian.dynlacht@med.nyu.edu

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Abbreviations

ChIP	chromatin immunoprecipitation
MEFs	mouse embryonic fibroblasts
pRB	retinoblastoma protein

Introduction

Since the discovery of E2F nearly two decades ago as a biochemical activity able to bind the Adenovirus E2 promoter and control expression of genes involved in S phase, a considerable amount of information has accumulated in support of a pivotal role for this protein family in the temporal control of gene expression during the cell cycle. Since that time, E2F has grown from a single activity to more than ten distinct polypeptides, and it is now clear that E2F controls the expression of genes involved in diverse processes at different cell-cycle stages [1].

Why are there so many E2F proteins, and how do they differ? Assuming that evolution proceeds by successive genetic innovations that prove successful and profitable [2], we must accept the notion that an E2F–Rb pathway that is so complex has its *raison d'être* and try to understand its detailed mechanisms of action. In this review, we focus on recent advances in this field, with an emphasis on work addressing the question of functional differences among E2F family members.

The E2F family: ever-expanding membership and function

The transcriptional activity known as E2F comprises several members (E2F and DP protein members) that, in various heterodimeric configurations, binds DNA and regulates the expression of a multitude of genes involved in many aspects of cell growth and proliferation (see [1,3,4] for recent reviews). E2F components regulate transcription of their target genes when bound to their promoters as dimers with a DP protein. E2F family members can be functionally grouped into activators (E2F1, E2F2 and E2F3a), or repressors (E2F3b, E2F4, E2F5, E2F6 and E2F7) of transcription. The function of E2F as transcriptional regulator is intimately linked to its association with 'pocket proteins', represented by the retinoblastoma tumor suppressor protein, pRB, and its relatives p107 and p130 (reviewed in [5]). Figure 1 illustrates the architecture of E2F and pocket proteins.

E2F6 was the first E2F member to be discovered that lacks both a *trans*-activation and pocket-protein binding domain [6–8]. Because it cannot activate transcription and seems to bind the same sequences as the other E2F family members, it is thought to repress its target genes by preventing binding of activator E2Fs to DNA and/or by recruiting polycomb group proteins, part of a well-known transcriptional repressor complex ([9,10•]; reviewed in [11]). The recent discovery of human and mouse E2F7 genes [12,13••,14] adds an additional level of complexity to an already extensive family of regulators. At the structural level, the similitude of E2F7 with other known E2Fs is limited to its DNA-binding domains, and here E2F7 is further distinguished from its relatives by possessing two DNA-binding domains rather than one. The integrity of both domains is required for efficient DNA binding. Moreover, at least *in vitro*, E2F7 does not depend on dimerization with DP proteins for DNA binding, and biochemical evidence suggests that it binds DNA as a homodimer. This radical departure from the conventional mode of E2F regulation awaits *in vivo* confirmation. E2F7 lacks *trans*-activation, pocket-protein-binding, cyclin A-binding, and marked box domains.

PREVENTING RE-REPLICATION OF CHROMOSOMAL DNA

J. Julian Blow and Anindya Dutta‡*

Abstract | To ensure its duplication, chromosomal DNA must be precisely duplicated in each cell cycle, with no sections left unreplicated, and no sections replicated more than once. Eukaryotic cells achieve this by dividing replication into two non-overlapping phases. During late mitosis and G1, replication origins are 'licensed' for replication by loading the minichromosome maintenance (Mcm) 2–7 proteins to form a pre-replicative complex. Mcm2–7 proteins are then essential for initiating and elongating replication forks during S phase. Recent data have provided biochemical and structural insight into the process of replication licensing and the mechanisms that regulate it during the cell cycle.

For the cell division cycle to produce two daughter cells that inherit a perfect copy of the genetic material that is originally present in the mother cell, it must accomplish two complex tasks: the chromosomal DNA must first be precisely replicated, with no errors, deletions or duplications, and then the two copies must be precisely segregated into the two daughter cells. The accuracy of these events is crucial to multicellular organisms, in which any changes to the genome potentially give rise to cancers that threaten the life of the entire organism. This review concentrates on the first of these linked problems: how eukaryotic cells ensure that their chromosomal DNA is precisely duplicated during the S phase of the cell cycle.

A large number of replication origins (typically ~10^3–10^5) (BOX 1) are used by eukaryotes to ensure that the entire genome is fully replicated. But these origins must be strictly regulated. How does the cell know whether it has already replicated a section of DNA in S phase? Eukaryotes have solved this problem by providing a marker to distinguish replicated from unreplicated DNA. The fact that replicated DNA differs from unreplicated DNA was first indicated by the classic cell-fusion experiments of Rao and Johnson[1]. In hybrids of G1 and G2 cells, the unreplicated G1 nucleus passes directly into S phase, whereas the DNA of the G2 nucleus (which has already been replicated) does not replicate again until after the hybrid cell has passed through mitosis. The G2 nucleus is therefore refractory to further DNA replication. Subsequent work that used cell-free extracts of *Xenopus laevis* eggs refined this idea and suggested a model whereby replication origins were 'licensed' for replication during late mitosis and G1, but the licence was removed as the DNA was replicated[2]. Dividing the process of DNA replication into two non-overlapping phases (one phase that is permissive for the licensing of DNA replication and a second phase that is permissive for the initiation of replication, but not for licensing) can potentially explain how cells ensure the precise duplication of chromosomal DNA in a single cell cycle.

Detailed experimental support for the licensing model has now been obtained, which indicates that it comprises four essential features[3,4]. First, replication origins are licensed by stably binding complexes of the minichromosome maintenance 2–7 proteins (Mcm2, Mcm3, Mcm4, Mcm5, Mcm6 and Mcm7). Mcm2–7 proteins form an essential component of the pre-replicative complex (pre-RC) of proteins that are found at replication origins during G1 phase. Second, the binding of Mcm2–7 proteins to origin DNA is essential for the origin to initiate a pair of replication forks. Third, the licensing of origins and the loading of Mcm2–7 onto DNA is restricted to late mitosis and G1 of the cell cycle. Fourth, Mcm2–7 proteins are displaced from origins as DNA replication is initiated, probably

**Wellcome Trust Biocentre, University of Dundee, Dundee DD1 5EH, UK.*
‡Department of Biochemistry and Molecular Genetics, University of Virginia School of Medicine, Charlottesville, Virginia 22908, USA.
Correspondence to J.J.B.
e-mail:
j.j.blow@dundee.ac.uk
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Correspondence and requests for materials should be addressed to R.A.F (richard.flavell@yale.edu) or C.D. (cdong@mdanderson.org).

Rb inactivation promotes genomic instability by uncoupling cell cycle progression from mitotic control

Eva Hernando[1]*, Zaher Nahlé[3]*†, Gloria Juan[1], Elena Diaz-Rodriguez[2], Miguel Alaminos[1]†, Michael Hemann[3], Loren Michel[2], Vivek Mittal[3], William Gerald[1], Robert Benezra[2], Scott W. Lowe[3] & Carlos Cordon-Cardo[1]

[1]*Department of Pathology and* [2]*Cancer Biology and Genetics Program, Memorial Sloan-Kettering Cancer Center New York, New York 10021, USA*
[3]*Cold Spring Harbor Laboratory 1 Bungtown Road, Cold Spring Harbor, New York 11724, USA*

* These authors contributed equally to this work.
† Present addresses: Department of Internal Medicine, Washington University in St. Louis, Saint Louis, Missouri, Missouri 63110, USA (Z.N.); Department of Histology, School of Medicine, University of Granada, Avenida de Madrid, 11. E-18012 Granada, Spain (M.A.)

Advanced human cancers are invariably aneuploid, in that they harbour cells with abnormal chromosome numbers[1,2]. However, the molecular defects underlying this trait, and whether they are a cause or a consequence of the malignant phenotype, are not clear. Mutations that disable the retinoblastoma (Rb) pathway are also common in human cancers[1]. These mutations promote tumour development by deregulating the E2F family of transcription factors leading to uncontrolled cell cycle progression[3]. We show that the mitotic checkpoint protein Mad2 is a direct E2F target and, as a consequence, is aberrantly expressed in cells with Rb pathway defects. Concordantly, Mad2 is overexpressed in several tumour types, where it correlates with high E2F activity and poor patient prognosis. Generation of Rb pathway lesions in normal and transformed cells produces aberrant Mad2 expression and mitotic defects leading to aneuploidy, such that elevated Mad2 contributes directly to these defects. These results demonstrate how chromosome instability can arise as a by-product of defects in cell cycle control that compromise the accuracy of mitosis, and suggest a new model to explain the frequent appearance of aneuploidy in human cancer.

The appearance of aneuploidy in human cancers has been linked to defects in several processes[1,2]. Many studies suggest that aneuploidy arises from defects in the conserved spindle checkpoint that normally governs progression through mitosis[4,5]. In response to lack of tension or improper microtubule attachment at the kinetochores, a group of sensor proteins (Bub3, Bub1, BubR1, Mps1 and Mad2) releases a diffusible signal that inhibits the ubiquitin ligase activity of the anaphase promoting complex (APC) or cyclosome[6]. APC/cyclosome function is required for sister chromatid separation and cytokinesis[7]. Although mitotic checkpoint defects are often observed in cancer cells challenged with microtubule poisons, and inactivation of checkpoint components produces aneuploidy in yeast and mammalian cells[8–10], loss-of-function mutations in spindle checkpoint genes are rarely observed in human tumours[11,12].

Adenovirus E1A is a potent viral oncoprotein that acts, in part, by inactivating the Rb gene product and deregulating the E2F transcription factors[3]. In performing a series of microarray experiments, we observed that E1A significantly increased the expression of *MAD2* (Z.N., V.M. and S.W.L., unpublished observations), a crucial component of the spindle checkpoint that associates with the APC/cyclosome, and prevents its activation[7,13]. Northern and western blot analyses of *MAD2* transcript and protein confirmed its upregulation by E1A in mouse embryo fibroblasts (MEFs) (Fig. 1a, compare lanes 1 and 2 or 3 and 4, respectively). Similarly, MEFs isolated from $Rb^{-/-}$ mice displayed increased Mad2 levels compared to $Rb^{+/+}$ controls (Fig. 1a, compare lanes 5 and 6). IMR90 human fibroblasts expressing either *E1A* (Fig. 1b, compare lanes 1 and 2) or *E2F-1* (Fig. 1b, compare lanes 1 and 3) also expressed elevated Mad2 levels, implying that deregulation of the Rb/E2F pathway induces Mad2 through a conserved mechanism.

The above results are consistent with the possibility that *MAD2* is an E2F target. Concordantly, previous global chromatin immunoprecipitation/microarray studies suggested that E2Fs can bind the *MAD2* promoter in normal fibroblasts[14]. Indeed, analysis of the *MAD2* genomic sequence showed several putative E2F-binding sites in the *MAD2* promoter (see Supplementary Fig. S1), and subsequent chromatin immunoprecipitation (ChIP) analysis confirmed *in vivo* binding of E2F-1 to their predicted sites in *E1A*-expressing cells (Fig. 1c, lane 2). Moreover, a *MAD2* genomic fragment containing the E2F sites (Supplementary Fig. S1) conferred E2F responsiveness to a luciferase reporter following transient transfection into IMR90 (data not shown) or U-2OS cells (Fig. 1d) in a manner comparable to the *caspase-7* promoter, an established E2F target[15]. Therefore, *MAD2* is a physiological transcriptional target of E2F.

Many E2F targets are cell-cycle regulated[3]. Therefore, we examined the cell cycle distribution of Mad2 expression in normal synchronously and asynchronously cycling cells. The level of Mad2 was undetectable in quiescent IMR90 cells (Fig. 1e, $t = 0$) and increased after cells entered S phase following serum addition, reaching maximum levels in G2/M, albeit slightly after cyclin A. The increase in Mad2 was confined largely to mitosis, as assessed by co-expression with phosphorylated histone H3 following release from a double-thymidine-induced S-phase arrest (Fig. 1f), and by laser scanning cytometry (LSC) analysis of Mad2 expression in unsynchronized NIH-3T3 fibroblasts (Fig. 1g). Presumably, Mad2 expression is regulated during the normal cell cycle as part of a

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Rb-Mediated Heterochromatin Formation and Silencing of E2F Target Genes during Cellular Senescence

Masashi Narita,[1] Sabrina Nuñez,[1,2] Edith Heard,[3] Masako Narita,[1] Athena W. Lin,[1,4] Stephen A. Hearn,[1] David L. Spector,[1] Gregory J. Hannon,[1] and Scott W. Lowe[1,*]

[1]Cold Spring Harbor Laboratory
1 Bungtown Road
Cold Spring Harbor, New York 11724

[2]Graduate Program in Genetics
State University of New York at Stony Brook
Stony Brook, New York 11794

[3]Centre National de la Recherche Scientifique UMR 218
Curie Institute
26 rue d'Ulm
75248 Paris Cedex 05
France

Summary

Cellular senescence is an extremely stable form of cell cycle arrest that limits the proliferation of damaged cells and may act as a natural barrier to cancer progression. In this study, we describe a distinct heterochromatic structure that accumulates in senescent human fibroblasts, which we designated senescence-associated heterochromatic foci (SAHF). SAHF formation coincides with the recruitment of heterochromatin proteins and the retinoblastoma (Rb) tumor suppressor to E2F-responsive promoters and is associated with the stable repression of E2F target genes. Notably, both SAHF formation and the silencing of E2F target genes depend on the integrity of the Rb pathway and do not occur in reversibly arrested cells. These results provide a molecular explanation for the stability of the senescent state, as well as new insights into the action of Rb as a tumor suppressor.

Introduction

Cellular senescence was originally described as the process of cell cycle arrest that accompanies the exhaustion of replicative potential in cultured human fibroblasts (Hayflick, 1965). Senescent cells remain metabolically active; display characteristic changes in cell morphology, physiology, and gene expression; and typically upregulate a senescence-associated β-galactosidase (SA-β-gal) activity (Campisi, 2001; Dimri et al., 1995; Shelton et al., 1999). Senescent cells are unable to express genes required for proliferation, even in a promitogenic environment (Dimri et al., 1994, 1996). These features distinguish senescence from quiescence, a nonproliferative state that is readily reversed in response to mitogens. Although "replicative" senescence is triggered by telomere attrition, an identical endpoint (often called "premature senescence" or "stasis") can be acutely produced in response to activated oncogenes, DNA damage, oxidative stress, and suboptimal cell culture conditions. These observations imply that cellular senescence, like apoptosis, is a cellular response to stress that limits the proliferation of damaged cells (Campisi, 2001; Mathon and Lloyd, 2001).

Although cellular senescence is typically studied in cultured cells, the process may be important in aging and cancer (Campisi, 2001). Cellular senescence is often considered a cellular counterpart of organismal aging and, indeed, increases in SA-β-gal activity can be detected in cells from older individuals and patients with premature aging syndromes. Moreover, mutations that prevent DNA repair or promote chronic DNA damage can promote premature senescence in vitro and aging in vivo, and some genes that modulate senescence in cultured cells also affect lifespan in mice. Owing to its antiproliferative effects, senescence also appears to be a potent antitumor mechanism. Hence, mutations in certain tumor suppressor genes compromise senescence, thereby contributing to cell immortalization and cancer. Furthermore, cytotoxic agents used in cancer chemotherapy can induce cellular senescence, and defects in this process promote drug resistance in vivo (Chang et al., 1999; Schmitt et al., 2002; te Poele et al., 2002).

The Rb and p53 tumor suppressors are important senescence regulators. Rb and p53 are typically activated during senescence, and enforced expression of either protein induces senescence in some cell types (Ferbeyre et al., 2002; Lee et al., 2000). In human fibroblasts, DNA tumor virus oncoproteins that interfere with Rb and p53 function can bypass senescence. For example, SV40 large T antigen binds both Rb and p53 and overcomes replicative senescence, whereas large T mutants defective in binding either protein are less able to do so (Shay et al., 1991). Similarly, adenovirus E1A targets the Rb family and interferes with p53-mediated arrest and prevents senescence induced by oncogenic *ras* and DNA damaging agents (Lowe and Ruley, 1993; Serrano et al., 1997). In mouse embryo fibroblasts (MEFs), p53 loss is sufficient to overcome senescence, whereas inactivation of Rb alone has no obvious effect (Lowe and Sherr, 2003). Nevertheless, the Rb family contributes to senescence in this cell type, since cells lacking Rb along with the related p107 and p130 proteins fail to senesce in culture (Dannenberg et al., 2000; Sage et al., 2000).

In many instances, p53 and Rb are activated to promote senescence by products of the *INK4a/ARF* locus (Lowe and Sherr, 2003). This locus encodes two tumor suppressors, $p16^{INK4a}$ and $p14^{ARF}$ ($p19^{ARF}$ in mice), expressed from partially overlapping nucleotide sequences read in alternative reading frames. $p16^{INK4a}$ engages the Rb pathway by inhibiting cyclin D-dependent kinases that would otherwise phosphorylate and inactivate Rb. In contrast, $p14^{ARF}$ increases the growth suppressive functions of p53 by interfering with its negative regulator, Mdm2. Both $p16^{INK4a}$ and $p14^{ARF}$ accumulate in senescent cells and can promote senescence when

*Correspondence: lowe@cshl.edu
[4]Present address: Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, New York 14263.

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A Senescence Program Controlled by p53 and p16^{INK4a} Contributes to the Outcome of Cancer Therapy

Clemens A. Schmitt,[1,4] Jordan S. Fridman,[1] Meng Yang,[2] Soyoung Lee,[1,4] Eugene Baranov,[2] Robert M. Hoffman,[2] and Scott W. Lowe[1,3]
[1]Cold Spring Harbor Laboratory
1 Bungtown Road
Cold Spring Harbor, New York 11724
[2]AntiCancer, Inc.
7917 Ostrow Street
San Diego, California 92111

Summary

***p53* and *INK4a/ARF* mutations promote tumorigenesis and drug resistance, in part, by disabling apoptosis. We show that primary murine lymphomas also respond to chemotherapy by engaging a senescence program controlled by p53 and p16^{INK4a}. Hence, tumors with *p53* or *INK4a/ARF* mutations—but not those lacking *ARF* alone—respond poorly to cyclophosphamide therapy in vivo. Moreover, tumors harboring a Bcl2-mediated apoptotic block undergo a drug-induced cytostasis involving the accumulation of p53, p16^{INK4a}, and senescence markers, and typically acquire *p53* or *INK4a* mutations upon progression to a terminal stage. Finally, mice bearing tumors capable of drug-induced senescence have a much better prognosis following chemotherapy than those harboring tumors with senescence defects. Therefore, cellular senescence contributes to treatment outcome in vivo.**

Introduction

Chemotherapy remains the primary treatment for systemic malignancies. However, some tumors are inherently insensitive to chemotherapeutic agents and others acquire resistance upon relapse. Most conventional agents damage cellular components, often DNA, and for years it was assumed that this damage was directly responsible for their anti-tumor effect (Johnstone et al., 2002). Consequently, drug resistance was thought to arise primarily from changes that prevented the drug-target interaction, including overexpression of drug efflux pumps (e.g., P-glycoprotein) or intracellular detoxifiers (e.g., glutathione). It is now clear that drug-induced damage is not invariably lethal, but can instead initiate a series of post-damage responses including apoptosis, cell-cycle checkpoints, mitotic catastrophe, and cellular senescence (Chang et al., 1999; Johnstone et al., 2002). Accordingly, the integrity of these damage responses might also influence treatment sensitivity.

Apoptosis is a well-characterized post-damage program that contributes to drug action (Johnstone et al., 2002). Diverse anticancer agents can induce apoptosis through common pathways and, consequently, mutations that disable these pathways can confer multidrug resistance (Lowe et al., 1993). For example, the Bcl2 oncoprotein is a potent suppressor of apoptosis that can produce multidrug resistance in cultured cells and animal models, and Bcl2 overexpression correlates with poor treatment outcome in some clinical settings (Johnstone et al., 2002). Nevertheless, despite extensive efforts, the overall contribution of apoptotic defects to clinical drug resistance has been difficult to assess.

Many of the genes that control apoptosis during tumor development can also influence treatment sensitivity. For example, the p53 tumor suppressor promotes apoptosis in response to stress-inducing stimuli, and, in turn, p53 inactivation facilitates tumor development (Vogelstein et al., 2000). Anticancer agents also activate p53 to promote apoptosis, and loss of p53 function can promote drug resistance in cultured cells and animal models (e.g., Lowe et al., 1993; Schmitt et al., 1999). Hence, *p53* mutations can simultaneously account for a survival advantage during tumor development and inherent resistance to chemotherapeutic drugs. Of note, p53 can also engage several cell-cycle checkpoints and trigger cellular senescence (Bunz et al., 1998; Chang et al., 1999; Kastan et al., 1991; Suzuki et al., 2001; Waldman et al., 1997), although the extent to which these processes contribute to drug action in vivo is not known.

Like *p53*, the *INK4a/ARF* locus is frequently mutated in human cancers and has been linked to treatment sensitivity. This locus encodes two tumor suppressors—ARF and p16^{INK4a}—that share coding sequence translated in different reading frames (Sherr, 2001a). ARF can activate p53 by interfering with its negative regulator Mdm2. Most studies show that ARF is not induced by DNA damage but can potentiate a DNA damage response (de Stanchina et al., 1998; Kamijo et al., 1999). Instead, ARF is induced by mitogenic oncogenes, perhaps as part of a failsafe mechanism that counters hyperproliferative signals (Sherr, 2001a). By contrast, p16^{INK4a} is a cyclin-dependent kinase inhibitor that promotes cell-cycle arrest via the Rb tumor suppressor pathway. *INK4a* regulation is poorly understood, but its expression increases with the onset of cellular senescence (Alcorta et al., 1996; Hara et al., 1996; Robles and Adami, 1998; Serrano et al., 1997). Mutations affecting both *INK4a* and *ARF* are common in malignant tumors, although disruption of either gene alone promotes tumorigenesis in mice and humans (Ruas and Peters, 1998; Sherr, 2001b). *INK4a/ARF* deletions correlate with poor treatment outcome in patients and in mouse models (Maloney et al., 1999; Schmitt et al., 1999), although the precise contribution of each gene product to treatment sensitivity has not been examined.

The determinants of drug action are typically studied in tumor-derived cell lines treated in culture or as xenografts. Animal models that recapitulate the genetics and pathobiology of human malignancies provide powerful alternatives, since these systems are experimentally tractable yet utilize spontaneous tumors treated at their natural site. *Eμ-myc* transgenic mice overexpress the

[3]Correspondence: lowe@cshl.edu
[4]Present address: Max-Delbrück-Center for Molecular Medicine and Charité/Campus Virchow-Hospital, Department of Hematology/Oncology, Humboldt University, D-13353 Berlin, Germany.

Induction of apoptosis by an inhibitor of the mitotic kinesin KSP requires both activation of the spindle assembly checkpoint and mitotic slippage

Weikang Tao,[1,*] Victoria J. South,[1] Yun Zhang,[1] Joseph P. Davide,[1] Linda Farrell,[1] Nancy E. Kohl,[1] Laura Sepp-Lorenzino,[1] and Robert B. Lobell[1]

[1]Department of Cancer Research, Merck Research Laboratories, West Point, Pennsylvania 19486
*Correspondence: weikang_tao@merck.com

Summary

The inhibition of KSP causes mitotic arrest by activating the spindle assembly checkpoint. While transient inhibition of KSP leads to reversible mitotic arrest, prolonged exposure to a KSP inhibitor induces apoptosis. Induction of apoptosis by the KSP inhibitor couples with mitotic slippage. Slippage-refractory cells show resistance to KSP inhibitor-mediated lethality, whereas promotion of slippage after mitotic arrest enhances apoptosis. However, attenuation of the spindle checkpoint confers resistance to KSP inhibitor-induced apoptosis. Furthermore, sustained KSP inhibition activates the proapoptotic protein, Bax, and both activation of the spindle checkpoint and subsequent mitotic slippage are required for Bax activation. These studies indicate that in response to KSP inhibition, activation of the spindle checkpoint followed by mitotic slippage initiates apoptosis by activating Bax.

Introduction

The mitotic spindle is a pharmaceutically validated target for cancer therapeutics (Wood et al., 2001). Antispindle agents, such as taxanes and vinca alkaloids, which interfere with microtubule dynamics by targeting tubulin, have been widely used in the clinic for the treatment of human malignancies (Jordan and Wilson, 2004). However, since microtubules are not only essential for mitosis, but also required for other critical physiological functions, such as intracellular transport and organelle positioning, the microtubule inhibitors act on both proliferating and postmitotic cells and exhibit microtubule-dependent side effects, including peripheral neuropathy (Rowinsky et al., 1993; Tuxen and Hansen, 1994). Thus, agents that target the mitotic spindle via a novel mechanism of action and with greater specificity toward tumors are desired for the treatment of human neoplasm.

KSP (hsEg5, kinesin-5) is a mitotic spindle motor protein belonging to the kinesin superfamily (Vale and Fletterick, 1997; Dagenbach and Endow, 2004) that plays an essential role in centrosome separation and in the formation of a bipolar mitotic spindle (Enos and Morris, 1990; Blangy et al., 1995; Sawin and Mitchison, 1995). Inhibition of KSP function led to cell cycle arrest in mitosis with the formation of monopolar mitotic spindles (Blangy et al., 1995; Mayer et al., 1999; Kapoor et al., 2000). A small molecule inhibitor of KSP exhibited antitumor activity superior to that of paclitaxel in a human tumor xenograft model (Sakowicz et al., 2004). Since KSP functions exclusively in mitosis, inhibitors of this mitotic kinesin should lack the liability of tubulin-targeting agents and act specifically on proliferating cells. In addition, they should be effective in taxane-resistant tumors, where the resistance results from mutations on β-tubulin (Monzo et al., 1999) or alterations in the expression of tubulin isoforms (Hasegawa et al., 2003). Indeed, KSP inhibitors have recently entered clinical trials for cancer therapy.

Since KSP inhibitors are being developed as a new generation of antimitotic agents that antagonize a novel target, an in-depth understanding of their biological mechanism of action in cancer cells is warranted and could provide insights into the effective development of these agents in the clinic. Early studies in *Xenopus* egg extracts and mammalian cells showed that the suppression of KSP activity induced the formation of monoastral spindles and provoked the activation of the spindle assembly checkpoint (Sawin et al., 1992; Blangy et al., 1995; Mayer et al., 1999; Kapoor et al., 2000). The spindle assembly checkpoint or mitotic checkpoint is a signaling pathway with multiple components, including Mps1, Bub1, BubR1, Bub3,

SIGNIFICANCE

KSP inhibitors are novel antimitotic agents that have entered clinical trials for cancer therapy. We show that sustained activation of the spindle checkpoint by a KSP antagonist, followed by mitotic slippage, activates Bax and initiates apoptosis. Both spindle checkpoint-deficient and mitotic slippage-refractory cells are resistant to KSP inhibitor-induced Bax activation and apoptosis. Sequential suppression of Cdk1 synergizes with the KSP inhibitor in inducing cell death by facilitating mitotic slippage in spindle checkpoint-competent cells. These results delineate the critical events that mediate the lethality of KSP inhibitors and provide clues on the identification of KSP inhibitor-resistant tumors and the selection of adjunct agents that may enhance the efficacy of KSP inhibitors.

Cytoplasmic localization of p21$^{Cip1/WAF1}$ by Akt-induced phosphorylation in *HER-2/neu*-overexpressing cells

Binhua P. Zhou, Yong Liao, Weiya Xia, Bill Spohn, Mong-Hong Lee and Mien-Chie Hung*

Department of Molecular and Cellular Oncology, Breast Cancer Basic Research Program, University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, USA
**e-mail: mhung@notes.mdacc.tmc.edu*

Amplification or overexpression of *HER-2/neu* in cancer cells confers resistance to apoptosis and promotes cell growth. The cellular localization of p21$^{Cip1/WAF1}$ has been proposed to be critical either in promoting cell survival or in inhibiting cell growth. Here we show that *HER-2/neu*-mediated cell growth requires the activation of Akt, which associates with p21$^{Cip1/WAF1}$ and phosphorylates it at threonine 145, resulting in cytoplasmic localization of p21$^{Cip1/WAF1}$. Furthermore, blocking the Akt pathway with a dominant-negative Akt mutant restores the nuclear localization and cell-growth-inhibiting activity of p21$^{Cip1/WAF1}$. Our results indicate that *HER-2/neu* induces cytoplasmic localization of p21$^{Cip1/WAF1}$ through activation of Akt to promote cell growth, which may have implications for the oncogenic activity of *HER-2/neu* and Akt.

The *HER-2/neu* gene (also known as *c-erbB2*) encodes a transmembrane receptor tyrosine kinase of relative molecular mass 185,000 (M_r 185K) that shares partial homology with the other members of the family of epidermal growth-factor receptors. Amplification or overexpression of *HER-2/neu* occurs in ~30% of human breast and ovarian cancers and is a marker of poor prognosis[1–3]. We have previously shown that *HER-2/neu* activates the phosphatidylinositol-3 kinase (PI-3K)/Akt pathway and confers resistance to apoptosis induced by tumour-necrosis factor[4]. The PI-3K/Akt pathway has an important role in preventing cells from undergoing apoptosis and contributes to the pathogenesis of malignancy[5,6]. For example, activated Akt phosphorylates specific targets such as Bad[7], caspase-9 (ref. 8), forkhead transcription factors[9,10] and IKK-α (refs 11, 12), thereby promoting cell survival. However, in addition to its anti-apoptotic function, Akt is also involved in cell proliferation[13–15]. Furthermore, Akt detaches from the inner surface of the plasma membrane, where it is initially activated, and relocalizes to the nucleus within 30 min of activation by growth factors[16,17]. These findings indicate that some critical Akt targets that control cell-cycle progression may be located within the nucleus.

Cell-cycle progression is tightly regulated by the family of cyclin-dependent kinase (CDK) inhibitors. p21$^{Cip1/WAF1}$ was identified through its interaction with Cdk2 (ref. 18), and its expression is induced by activation of wild-type p53 (ref. 19), and during cellular senescence[20]. The cell-growth-inhibiting activity of p21$^{Cip1/WAF1}$ is strongly correlated with its nuclear localization[21,22]. However, recent evidence has shown that p21$^{Cip1/WAF1}$ can also localize in the cytoplasm and has an important role in protecting cells against apoptosis. For instance, nuclear p21$^{Cip1/WAF1}$ translocates to the cytoplasm after differentiation of U937 cells into monocytes, and this translocation event is accompanied by resistance to various apoptotic stimuli[23]. Furthermore, cytoplasmic p21$^{Cip1/WAF1}$ forms a complex with apoptosis-signal-regulating kinase 1 (ASK1) that inhibits the stress-induced mitogen-activated protein (MAP) kinase cascade and therefore results in resistance to apoptosis in these cells[23]. However, the mechanism that regulates the localization of p21$^{Cip1/WAF1}$ is still unknown.

Here we show that blocking the Akt pathway by using a dominant negative Akt mutant (DN-Akt) inhibits cell growth. This growth inhibition is correlated with nuclear localization of p21$^{Cip1/WAF1}$. We demonstrate that Akt can associate with p21$^{Cip1/WAF1}$ and phosphorylates a consensus threonine residue (T145) in the nuclear-localization signal (NLS) of p21$^{Cip1/WAF1}$, leading to the cytoplasmic localization of p21$^{Cip1/WAF1}$. We thus identify a new signalling pathway and show that overexpression of *HER-2/neu* may enhance cell proliferation by inducing cytoplasmic localization of p21$^{Cip1/WAF1}$ through the serine/threonine kinase Akt.

Results

The Akt pathway is required for *HER-2/neu*-mediated cell proliferation. To study the effect of Akt on *HER-2/neu*-mediated cell proliferation, we used a model system that consists of NIH3T3 cells, *HER-2/neu* 3T3 cells (NIH3T3 cells transformed with *HER-2/neu*) and DN-Akt 3T3 cells (*HER-2/neu* 3T3 cells transfected with DN-Akt)[4]. As expected, *HER-2/neu* 3T3 cells grew much faster than the parental NIH3T3 cells (Fig. 1a). The Akt pathway is known to be constitutively activated in *HER-2/neu* 3T3 cells[4], and when this pathway was blocked by DN-Akt, cell growth became slower (Fig. 1a). This was not due to the heterogeneity of the cell clones, because a specific PI-3K inhibitor, wortmannin, also produced a similar slowing of growth in *HER-2/neu* 3T3 cells (Fig. 1a). When the DNA synthesis rate was determined by measuring incorporation of [^{3}H]thymidine, *HER-2/neu* 3T3 cells also exhibited greater amounts of DNA synthesis than the parental NIH3T3 cells (Fig. 1b). *HER-2/neu*-induced DNA synthesis was significantly inhibited by blocking the Akt pathway with either wortmannin or DN-Akt.

As the net rate of cell growth depends on a fine balance between the rates of cell proliferation and cell death, we also investigated whether apoptosis contributes to the difference in growth in these cells. There was no significant difference in apoptosis among these cells, as measured by fluorescence-activated cell sorting (FACS) analysis (Fig. 1c). Therefore, the reduction in cell growth in DN-Akt 3T3 cells was most probably a result of the reduction in cell proliferation. To investigate further, we also carried out the same experiments using another *HER-2/neu*-overexpressing breast-cancer cell line, MDA-MB453, and stable DN-Akt transfectants of it[4]. As in *HER-2/neu* 3T3 and DN-Akt 3T3 cells, three independent clones of DN-Akt transfectants showed reductions in cell growth and DNA synthesis (Fig. 1d, e), but no difference was observed in apoptosis (FACS analysis, data not shown). A revertant that had lost DN-Akt during culture exhibited rates of cell growth and DNA synthesis that were almost identical to those of parental MDA-

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ARF-BP1/Mule Is a Critical Mediator of the ARF Tumor Suppressor

Delin Chen,[1] Ning Kon,[1] Muyang Li,[1] Wenzhu Zhang,[1] Jun Qin,[2] and Wei Gu*
[1]Institute for Cancer Genetics
Department of Pathology
College of Physicians and Surgeons
Columbia University
1150 St. Nicholas Avenue
New York, New York 10032
[2]Departments of Biochemistry and Cell Biology
Baylor College of Medicine
One Baylor Plaza
Houston, Texas 77030

Summary

Although the importance of the ARF tumor suppressor in p53 regulation is well established, numerous studies indicate that ARF also suppresses cell growth in a p53/Mdm2-independent manner. To understand the mechanism of ARF-mediated tumor suppression, we identified a ubiquitin ligase, ARF-BP1, as a key factor associated with ARF in vivo. ARF-BP1 harbors a signature HECT motif, and its ubiquitin ligase activity is inhibited by ARF. Notably, inactivation of ARF-BP1, but not Mdm2, suppresses the growth of p53 null cells in a manner reminiscent of ARF induction. Surprisingly, in p53 wild-type cells, ARF-BP1 directly binds and ubiquitinates p53, and inactivation of endogenous ARF-BP1 is crucial for ARF-mediated p53 stabilization. Thus, our study modifies the current view of ARF-mediated p53 activation and reveals that ARF-BP1 is a critical mediator of both the p53-independent and p53-dependent tumor suppressor functions of ARF. As such, ARF-BP1 may serve as a potential target for therapeutic intervention in tumors regardless of p53 status.

Introduction

The p53 protein has been described as a "guardian of the genome" because of its crucial role in coordinating cellular responses to stress (Lane, 1992; Levine, 1997). The antiproliferative effects of p53 are imparted through a variety of mechanisms that include cell cycle arrest, apoptosis, and cellular senescence/aging (Vogelstein et al., 2000; Lowe and Sherr, 2003). p53 can be thought of as the central node of a regulatory circuit that monitors signaling pathways from diverse sources, including DNA damage responses (e.g., ATM/ATR activation), abnormal oncogenic events (e.g., Myc or Ras activation), and everyday cellular processes (e.g., growth factor stimulation) (Giaccia and Kastan, 1998; Prives and Hall, 1999; Vousden and Lu, 2002; Brooks and Gu, 2003). While p53 mutations have been documented in more than half of all human tumors (Hollstein et al., 1999), defects in other components of the p53 pathway, such as the ARF tumor suppressor, are observed in tumor cells that retain wild-type p53 (Sherr, 2001; Sharpless and DePinho, 2004). Thus, inactivation of the p53 pathway appears to be a common, if not universal, feature of human cancer.

The cellular functions of p53 are rapidly activated in response to stress. Although the mechanisms of p53 activation are not fully understood, they are generally thought to entail posttranslational modifications of p53, mainly including ubiquitination, phosphorylation, and acetylation (Brooks and Gu, 2003; Giaccia and Kastan, 1998). Ubiquitination of p53 was first discovered in papillomavirus-infected cells, where p53 degradation is mediated by the viral E6 protein and a HECT domain-containing ubiquitin ligase called E6-AP (Munger and Howley, 2002). In normal cells, Mdm2, a RING finger oncoprotein, acts as a specific E3 ubiquitin ligase for p53 (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997; Fuchs et al., 1998; Fang et al., 2000), which, if malignantly activated, has the potential to counteract the tumor suppressor functions of p53 (Michael and Oren, 2003). The critical role of Mdm2 in regulating p53 is best illustrated by studies carried out in mice where inactivation of p53 was shown to completely rescue the embryonic lethality caused by loss of Mdm2 function (Jones et al., 1995; Montes de Oca Luna et al., 1995).

Although earlier studies suggested that Mdm2 is the primary factor controlling p53 turnover, there is growing evidence that p53 degradation is more complex than originally anticipated. We recently found that Mdm2 differentially catalyzes either monoubiquitination or polyubiquitination of p53 in a dosage-dependent manner (Li et al., 2003). It seems likely that these distinct mechanisms are exploited in different physiological settings. For example, Mdm2-mediated polyubiquitination and nuclear degradation may play a critical role in suppressing p53 function during the latter stages of a DNA damage response or when Mdm2 is malignantly overexpressed (Xirodimas et al., 2001a; Shirangi et al., 2002). On the other hand, Mdm2-mediated monoubiquitination and subsequent cytoplasmic translocation of p53 may represent an important means of p53 regulation in unstressed cells, where Mdm2 is maintained at low levels (Freedman et al., 1999; Stommel et al., 1999; Boyd et al., 2000; Geyer et al., 2000). Moreover, deubiquitination of either p53 or Mdm2 by HAUSP is apparently a critical event in these dynamic processes (Li et al., 2004; Cummins et al., 2004), and additional cellular factors are necessary to facilitate p53 degradation in normal cells. Indeed, it was recently reported that the ubiquitin ligases COP1 and Pirh2 are directly involved in p53 degradation (Leng et al., 2003; Dornan et al., 2004). Taken together, these studies suggest that, while Mdm2 is a key regulator of p53 function, p53 degradation is mediated through both Mdm2-dependent and Mdm2-independent pathways in vivo.

ARF (known as $p14^{ARF}$ in humans and $p19^{ARF}$ in mouse) was originally identified as an alternative transcript of the Ink4a/ARF tumor suppressor locus, a gene that encodes the $p16^{Ink4a}$ inhibitor of cyclin-dependent

*Correspondence: wg8@columbia.edu

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Negative Control of p53 by Sir2α Promotes Cell Survival under Stress

Jianyuan Luo,[1] Anatoly Y. Nikolaev,[1] Shin-ichiro Imai,[2] Delin Chen,[1] Fei Su,[1] Ariel Shiloh,[1] Leonard Guarente,[2] and Wei Gu[1,3]
[1]Institute of Cancer Genetics and Department of Pathology
College of Physicians & Surgeons
Columbia University
1150 St. Nicholas Avenue
New York, New York 10032
[2]Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Summary

The NAD-dependent histone deacetylation of Sir2 connects cellular metabolism with gene silencing as well as aging in yeast. Here, we show that mammalian Sir2α physically interacts with p53 and attenuates p53-mediated functions. Nicotinamide (Vitamin B3) inhibits an NAD-dependent p53 deacetylation induced by Sir2α, and also enhances the p53 acetylation levels in vivo. Furthermore, Sir2α represses p53-dependent apoptosis in response to DNA damage and oxidative stress, whereas expression of a Sir2α point mutant increases the sensitivity of cells in the stress response. Thus, our findings implicate a p53 regulatory pathway mediated by mammalian Sir2α. These results have significant implications regarding an important role for Sir2α in modulating the sensitivity of cells in p53-dependent apoptotic response and the possible effect in cancer therapy.

Introduction

The p53 tumor suppressor exerts anti-proliferative effects, including growth arrest, apoptosis, and cell senescence, in response to various types of stress (Levine, 1997; Prives and Hall, 1999; Vogelstein et al., 2000). Mutations within the *p53* gene have been well documented in more than half of all human tumors. Accumulating evidence further indicates that, in the cells that retain wild-type p53, other defects in the p53 pathway also play an important role in tumorigenesis (Prives and Hall, 1999). The molecular function of p53 that is required for tumor suppression involves its ability to act as a transcriptional factor in regulating downstream target gene expression (reviewed in Nakano and Vousden, 2001; Yu et al., 2001).

p53 is a short-lived protein whose activity is maintained at low levels in normal cells. Tight regulation of p53 is essential for its effect on tumorigenesis as well as maintaining normal cell growth. The precise mechanism by which p53 is activated by cellular stress is not completely understood; it is generally thought to involve mainly posttranslational modifications of p53, including phosphorylation and acetylation (reviewed in Appella and Anderson, 2000). Early studies demonstrated that CBP/p300, a *h*istone *a*cetyl-*t*ransferase (HAT), acts as a coactivator of p53 and potentiates its transcriptional activity as well as biological function in vivo (Gu et al., 1997; Lill et al., 1997; Avantaggiati et al., 1997). Significantly, the observation of functional synergism between p53 and CBP/p300 together with its intrinsic HAT activity led to the discovery of an FAT (transcriptional *f*actor *a*cetyl-*t*ransferase) activity of CBP/p300 on p53; this finding also predicted that acetylation may represent a general functional modification for nonhistone proteins in vivo (Gu and Roeder, 1997).

By developing site-specific acetylated p53 antibodies, CBP/p300 mediated acetylation of p53 was further confirmed in vivo by a number of studies (reviewed in Appella and Anderson, 2000). Significantly, the steady-state levels of acetylated p53 are stimulated in response to various types of stress, indicating the important role of p53 acetylation in stress response (reviewed in Ito et al., 2001). By introducing a transcriptional defective p53 mutant ($p53^{Q25S26}$) into mice, it was found that the mutant mouse thymocytes and ES cells failed in undergoing DNA damage-induced apoptosis (Chao et al., 2000; Jimenez et al., 2000). Interestingly, this mutant protein was phosphorylated normally at the N terminus in response to DNA damage but could not be acetylated at the C terminus (Chao et al., 2000), supporting a critical role of p53 acetylation in p53-dependent apoptotic response (Chao et al., 2000; Luo et al., 2000). Furthermore, it has been found that oncogenic Ras as well as PML can upregulate the levels of acetylated p53 in normal primary fibroblasts, and also induce premature senescence in a p53-dependent manner (Pearson et al., 2000, Ferbeyre et al., 2000). p53 acetylation may also play a critical role in protein stabilization (Rodriguez et al., 2000; Nakamura et al., 2000; Ito et al., 2001). In addition, another independent study showed that acetylation, but not phosphorylation of the p53 C terminus, may be required to induce metaphase chromosome fragility in the cell (Yu et al., 2000).

The yeast silent information regulator 2 (Sir2) protein belongs to a family of histone deacetylases (reviewed in Guarente, 2000; Shore, 2000). Sir2 activity is nicotinamide adenine dinucleotide (NAD)-dependent, but can not be inhibited by TSA (Imai et al., 2000; Landry et al., 2000a; Smith et al., 2000). The NAD-dependent deacetylase activity of Sir2 is essential for its functions, and this activity also connects its biological role with cellular metabolism in yeast (Guarente, 2000). Recently, mammalian Sir2 homologs have been found to also contain the NAD-dependent histone deacetylase activity (Imai et al., 2000; Smith et al., 2000), further supporting that the enzymatic activity is key to elucidate the molecular mechanism for its mediated functions. Among Sir2 and its homolog proteins (HSTs) in yeast, Sir2 is the only protein exclusively localized in nuclei, whose activity is critical for both gene silencing and extension of yeast life span (reviewed in Guarente, 2000). Based on protein sequence homology analysis, mouse Sir2α and its human ortholog SIRT1 (or human Sir2α) are the closest

[3]Correspondence: wg8@columbia.edu

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A Novel Human p53 Isoform Is an Essential Element of the ATR-Intra-S Phase Checkpoint

Gabor Rohaly, Jan Chemnitz, Silke Dehde, Alejandro Mena Nunez, Jochen Heukeshoven, Wolfgang Deppert, and Irena Dornreiter*
Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie
Universität Hamburg
Martinistrasse 52
D-20251 Hamburg
Germany

Summary

The archetypal human tumor suppressor p53 is considered to have unique transactivation properties. The assumption is based on the fact that additionally identified human p53 isoforms lack transcriptional activity. However, we provide evidence for the existence of an alternatively spliced p53 isoform (Δp53) that exerts its transcriptional activity independent from p53. In contrast to p53, Δp53 transactivates the endogenous *p21* and *14-3-3σ* but not the *mdm2*, *bax*, and *PIG3* promoter. Cell cycle studies showed that Δp53 displays its differential transcriptional activity only in damaged S phase cells. Upon activation of the ATR-intra-S phase checkpoint, Δp53, but not p53, transactivates the Cdk inhibitor *p21*. Induction of p21 results in downregulation of cyclin A-Cdk activity and accordingly attenuation of S phase progression. Data demonstrate that the Δp53-p21-cyclin A-Cdk pathway is crucial to facilitate uncoupling of repair and replication events, indicating that Δp53 is an essential element of the ATR-intra-S phase checkpoint.

Introduction

Activation of the tumor suppressor p53 after genotoxic insults leads to the induction of downstream events that provide a complex network of signals leading to cell cycle arrest or apoptosis (Vogelstein et al., 2000). Both events are in large part due to p53-dependent transcriptional activation of several downstream genes including cell cycle regulators (e.g., *p21*, *14-3-3σ*, *Gadd45*) and proapoptotic factors (e.g., *bax* and *PIGs*; El-Deiry, 1998). Since both pathways are activated by p53-mediated transactivation of genes, regulatory mechanisms must exist to determine the choice of the appropriate target genes within a given cellular and physiological context. On the level of the p53 protein, distinct post-translational modifications and/or binding to other proteins are most likely required to determine the promoter selectivity of p53 (Appella and Anderson, 2001; An et al., 2004). The complex regulatory web that mobilizes p53 after stress is continuously expanding and includes key checkpoint regulators such as the phosphatidylinositol 3-kinase family members ataxia telangiectasia mutated (ATM) and ATM-Rad3-related protein (ATR) as well as the downstream checkpoint kinases Chk2 and Chk1. Phosphorylation on serine (S) 20 of p53 by Chk2/Chk1 helps to stabilize p53 by uncoupling it from the Mdm2 ubiquitin ligase (Chehab et al., 2000; Hirao et al., 2000), while ATM/ATR-catalyzed phosphorylation on S-15 participates in the activation of p53 (Shiloh, 2001).

Additionally, it was reported that alternative splicing of *p53* is a candidate to regulate the promoter selectivity of p53 in mouse cells (Arai et al., 1986; Kulesz-Martin et al., 1994). However, analysis of various human cell lines failed to detect any equivalent functional C-terminal p53 splice variant (Rehberger et al., 1997; Will et al., 1995). Yet, an N-terminally truncated human p53-isoform (p47) has been identified, which is the product of alternative splicing of *p53* (Ghosh et al., 2004). The alternative splice-derived p47 product is able to control p53 ubiquitination, cell localization, and activity, but does not display transcriptional activity.

We now show that alternative splicing of *p53* generates a novel p53 isoform, designated as Δp53, that is present in primary and established primate cells of different tissue types. Our results reveal a novel pathway that operates in the UV-induced ATR-mediated intra-S phase checkpoint, which depends on the differential transcriptional activity of Δp53.

Results

Identification of a Novel p53 Splice Variant (Δp53) in Primate Cells

We used a highly sensitive, nonquantitative nested reverse transcription (RT)-PCR-based approach (Cooley and Bergtrom, 2001) to detect possible alternative splicing of primate *p53*. First, the cDNA was amplified between exon 4 and C-terminal exon 11 by using appropriate sense (E4F) and antisense (E11R) primers. Second, the PCR reaction was performed with nested PCR primers to exon 6/7 (E6/7F) and exon E11 (E11bR). Amplification of exons 6/7–11 generated the expected 520 bp PCR product and one additional 325 bp amplicon (Figure 1A). The two PCR products were detected in primary and established wild-type *p53* (wtp53) human and monkey cells of different tissue types (Figure 1A, lanes 1–6). Both amplicons were also found in mutant *p53* (mutp53) primate cells (Figure 1A, lanes 7 and 8), but not in the *p53* null cell line H1299 (Figure 1A, lane 9). Sequence analysis of both bands obtained from wtp53 cell lines CV-1 and HSC93 revealed that the major band (520 bp) represents the regularly spliced p53, whereas the lower molecular weight band (325 bp) results from alternative splicing. The alternative splice product designated as Δp53 lacks 198 nucleotides that are located in exons 7, 8, and 9. The deletion junction contains a donor-site-like splice cassette (CACTGGA) within the coding exon 7 (nucleotide 767) and an acceptor-site-like splice cassette (CACTGGA) within the coding exon 9 (nucleotide 965); the resulting 984 bp Δp53 transcript that contains the unique junction of exon 7 with 9 does not alter the open reading frame

*Correspondence: irene.dornreiter@hpi.uni-hamburg.de

Cell culture

CHO cells were grown in 100-mm dishes with F-12 medium (Life Technologies) supplemented with 10% fetal bovine serum. Non-synchronous cells were transiently transfected with 0.5 μg DNA per 35-mm well using Lipofectamine (Life Technologies). After 5 h, the transfection was stopped by switching to normal growth medium. Cells were collected 24 h later for immunoprecipitation or fixed for immunofluorescence.

Immunofluorescence staining

Transfected CHO cells with 70–80% confluence were fixed in 3.7% formaldehyde, 0.5% Triton X-100 in phosphate-buffered saline solution (PBS) at room temperature for 10 min. Post-extraction was performed in PBS containing 0.5% Triton X-100 for 5 min. Cells were washed in PBS with 0.1% Triton X-100 and then incubated in blocking solution (PBS with 20% normal goat serum) for 30 min. Cells transfected with Myc constructs were incubated with anti-Myc Cy3-labelled antibody, stained and fixed. Cells transfected with Flag constructs were incubated with anti-Flag (M2) and Cy3-anti-mouse antibody (Jackson Laboratories). Endogenous Dvl2 was visualized using goat anti-Dvl2 antibody (Santa Cruz) and blocking with 5% BSA instead of goat serum. Actin stress fibres and membranes were visualized using Alexa-488-phalloidin and Alexa-488-Con A (Molecular Probes), respectively. Samples were washed, mounted, analysed, and representative normal cells were selected using a fluorescence microscope equipped with a charge-coupled device camera (Nikon Eclipse TE300).

Immunoprecipitation and immunoblotting

Triton X-100-soluble aliquots from transfected CHO cells were prepared as described[8]. Actin binding was detected using goat polyclonal anti-actin antibody (Santa Cruz). The Myc- or Flag-tagged proteins were detected using anti-Myc (clone 9E10) and anti-Flag (M2) mouse monoclonal antibodies, respectively (Sigma). Primary antibodies were coupled to rabbit anti-goat (Jackson Laboratories) or horseradish peroxidase (HRP) donkey anti-mouse antibodies (Amersham). Polyvinylidene fluoride blots were then incubated with either HRP-conjugated anti-rabbit or HRP-conjugated anti-HRP antibodies (Amersham) and the signal visualized by enhanced chemiluminescence. For β-catenin detection, transfected CHO cells were lysed, and the soluble proteins were collected by centrifugation, separated by SDS–polyacrylamide gel electrophoresis, blotted, and reacted with anti-β-catenin antibody (Transduction Laboratories).

Phosphatase treatment

Triton X-100-soluble aliquots (5 μl) of Dvl2-transfected CHO cells were incubated at 30 °C for 1 h with 0.05 U of PP2A (Upstate) in 20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 60 mM β-mercapthoethanol, 1 mM $MgCl_2$, 2 mM EGTA, 100 μM $MnCl_2$ and 100 μg ml^{-1} BSA. Reactions were stopped by the addition of SDS sample buffer, resolved in Anderson gels[29], transferred and detected by western blotting.

Xenopus assays

In vitro RNA synthesis, *Xenopus laevis* embryo staging, injection, animal pole explants, polymerase chain reaction with reverse transcription (RT–PCR) and embryo extracts were performed as described[30].

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Correspondence and requests for materials should be addressed to M.O. (e-mail: michael.overduin@uchsc.edu).

Myc suppression of the *p21 Cip1* Cdk inhibitor influences the outcome of the p53 response to DNA damage

Joan Seoane, Hong-Van Le & Joan Massagué

Cell Biology Program and Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA

Activation of the tumour suppressor p53 by DNA damage induces either cell cycle arrest or apoptotic cell death[1]. The cytostatic effect of p53 is mediated by transcriptional activation of the cyclin-dependent kinase (CDK) inhibitor *p21 Cip1*, whereas the apoptotic effect is mediated by transcriptional activation of mediators including *PUMA* and *PIG3* (ref. 2). What determines the choice between cytostasis and apoptosis is not clear[3]. Here we show that the transcription factor Myc is a principal determinant of this choice. Myc is directly recruited to the *p21 Cip1* promoter by the DNA-binding protein Miz-1. This interaction blocks *p21 Cip1* induction by p53 and other activators. As a result Myc switches, from cytostatic to apoptotic, the p53-dependent

Specific protection against breast cancers by cyclin D1 ablation

Qunyan Yu, Yan Geng & Piotr Sicinski

Department of Cancer Biology, Dana-Farber Cancer Institute, and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115, USA

Breast cancer is the most common malignancy among women. Most of these cancers overexpress cyclin D1, a component of the core cell-cycle machinery. We previously generated mice lacking cyclin D1 using gene targeting. Here we report that these cyclin D1-deficient mice are resistant to breast cancers induced by the *neu* and *ras* oncogenes. However, animals lacking cyclin D1 remain fully sensitive to other oncogenic pathways of the mammary epithelium, such as those driven by c-*myc* or *Wnt-1*. Our analyses revealed that, in mammary epithelial cells, the Neu–Ras pathway is connected to the cell-cycle machinery by cyclin D1, explaining the absolute dependency on cyclin D1 for malignant transformation in this tissue. Our results suggest that an anti-cyclin D1 therapy might be highly specific in treating human breast cancers with activated Neu–Ras pathways.

Cyclin D1 belongs to the family of three closely related D-type cyclins, termed cyclin D1, D2 and D3. These three proteins are expressed in an overlapping, redundant fashion in all proliferating cell types. D-cyclins collectively control cell-cycle progression by activating their cyclin-dependent kinase partners, CDK4 and CDK6, which leads to phosphorylation of the retinoblastoma protein, and in turn to the advance through the G1 phase of the cell cycle[1].

Several lines of evidence point to an important role for cyclin D1 in breast cancer formation. The *cyclin D1* gene is amplified in up to 20% of human breast cancers[2], while cyclin D1 protein is overexpressed in over 50% of human mammary carcinomas[3–5]. The overexpression of cyclin D1 is seen in all histological types of human breast cancers[3]. It can be detected at the earliest stages of breast cancer progression, such as ductal carcinoma *in situ*, but not in premalignant lesions[6]. Once acquired, overexpression of cyclin D1 is maintained in all stages of the disease including the metastatic lesions[3,7]. Importantly, overexpression of cyclin D1 seems to have a causative role in breast cancer formation, as transgenic mice engineered to overexpress cyclin D1 in mammary glands succumb to breast cancers[8].

We and others previously generated cyclin D1-deficient mice[9,10]. We found that these *cyclin D1*$^{-/-}$ animals were viable and showed a narrow set of developmental abnormalities restricted to the retina and the nervous system[9,10]. In adult mice, however, loss of cyclin D1 had virtually no impact on mouse physiology, except that the mammary glands of *cyclin D1*$^{-/-}$ females failed to undergo full lobuloalveolar development during the late stage of pregnancy. This defect was restricted to pregnancy-associated proliferation, because *cyclin D1*$^{-/-}$ mice developed normal mammary glands during sexual maturation[9,10].

The limited impact of cyclin D1 loss on mouse physiology—together with the well documented role of cyclin D1 overexpression in human breast cancers—suggested to us that the ablation of cyclin D1 might be highly selective in shutting off the proliferation of breast-tumour cells while sparing other tissues. As a first step towards a potential strategy for anti-cyclin D1 therapy in human breast cancers, we investigated whether the ablation of cyclin D1 protects *cyclin D1*$^{-/-}$ mice against breast cancers.

To address this possibility, we crossed *cyclin D1*$^{-/-}$ mice with four different strains of breast-cancer-prone mouse mammary tumour virus (MMTV)-oncogene transgenic mice, and we generated *cyclin D1*$^{-/-}$/MMTV-oncogene animals. For our studies we used strains overexpressing the oncogenes v-Ha-*ras* (ref. 11), c-*neu* (ref. 12), c-*myc* (ref. 13) and *Wnt-1* (ref. 14).

Analyses of mammary glands

We started our analyses by determining the expression pattern of D-cyclins in mammary glands of non-transgenic, virgin wild-type and *cyclin D1*$^{-/-}$ females. Wild-type mammary glands expressed mostly cyclin D1, together with low levels of cyclin D2 and D3. Mammary glands of *cyclin D1*$^{-/-}$ females lacked cyclin D1, but instead contained modestly elevated levels of cyclin D2 and slightly increased levels of cyclin D3 (Fig. 1c). We presume that these low levels of cyclins D2 and D3 allow normal mammary development in *cyclin D1*$^{-/-}$ virgin mice.

We next compared the appearance of mammary glands of adult, virgin *cyclin D1*$^{-/-}$/MMTV-oncogene females with that of *cyclin D1*$^{+/+}$/MMTV-oncogene females. For each of four transgenic strains, we found that the appearance of *cyclin D1*$^{-/-}$ mammary glands was identical to that of wild-type mice (Fig. 1a). This is consistent with our earlier observations that *cyclin D1*$^{-/-}$ mice develop normal mammary glands in a virgin state[9]. For our tumour-susceptibility analyses, all females were kept as virgins throughout the entire observation period, except for the MMTV-*myc* mice (see Methods).

These control experiments provided us with an additional, unexpected observation. As reported previously[14], mammary glands of MMTV-*Wnt-1* transgenic mice undergo precocious lobuloalveolar development in a virgin state. As a result, mammary glands of MMTV-*Wnt-1* virgin females (and males) resemble those of pregnant wild-type, non-transgenic females[14]. Strikingly, we observed the same phenotype in *cyclin D1*$^{-/-}$/MMTV-*Wnt-1* mice (Fig. 1a). This suggests that Wnt-1-dependent proliferative signals do not require cyclin D1. This is in contrast with recent reports that the Wnt-1–β-catenin signalling pathway critically impinges on cyclin D1 (refs 15, 16). It also reveals that *cyclin D1*$^{-/-}$ mammary epithelium can undergo lobuloalveolar development under certain conditions.

Incidence of breast cancer

We observed MMTV-oncogene mice for breast cancer incidence. We found that the loss of cyclin D1 did not protect *cyclin D1*$^{-/-}$ mice from breast cancers induced by the *myc* and *Wnt-1* oncogenes (Fig. 2 and Table 1). In marked contrast, *cyclin D1*$^{-/-}$ mice were resistant to breast cancers induced by the *ras* and *neu* oncogenes. Thus, during the observation period, 19 of 21 *cyclin D1*$^{+/+}$/MMTV-*ras* mice died of breast cancers, developing a total of 39 tumours, whereas all 18 *cyclin D1*$^{-/-}$/MMTV-*ras* females remained free of tumours (Fig. 2 and Table 1). Likewise, 26 of 26 *cyclin D1*$^{+/+}$/MMTV-*neu* animals died of mammary carcinomas, developing a total of 79 tumours,

Immunoprecipitation and two-hybrid assays

Transfected cells were washed with PBS and treated with RIPA buffer with no SDS. Mouse monoclonal antibody against Flag (M2, Sigma) was added to cell extract and incubated overnight at 4 °C. Protein-A-conjugated Sepharose equilibrated in RIPA was then added and incubated for 2 h. The beads were extensively washed with ice-cold RIPA and the precipitate was dissolved in a sample buffer. Polyclonal antibody against p65 (Rockland) was used for western blot analysis. For mammalian two-hybrid experiments, the *ING4* gene was subcloned in-frame into pBIND and fused with the yeast GAL4 DNA-binding domain. p65 was subcloned in-frame into pACT and fused to the VP16 activation domain of herpes simplex virus. A series of constructs was used as controls: pBIND–Id and pACT–MyoD, containing GAL4–Id and VP16–MyoD fusion proteins, respectively, were used as positive controls; and pBIND and pACT, pBIND–ING4 and pACT, and pBIND and pACT–p65 were used as negative controls. Protein–protein interaction was determined by the activation of firefly luciferase.

Reporter gene assay

Cells (1×10^5 per well) were transfected with plasmids containing NF-κB-binding elements by Lipofectamine (Invitrogen). Two days after transfection, cells were lysed in Reporter Lysis Buffer (Promega), and luciferase activity was measured by a luminometer using an Enhanced Luciferase Assay kit (BD Biosciences).

Electrophoretic mobility shift assay

We carried out electrophoretic mobility shift assays (EMSAs) on nuclear extracts prepared as described[25] using the following double-stranded oligonucleotides: NF-κB, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; and nonspecific oligonucleotide (Oct-1), 5′-TGT CGA ATG CAA ATC ACT AGA A-3′. The oligonucleotide was 5′-end-labelled with biotin and annealed by standard procedures. Binding reaction was carried out by pre-incubating nuclear extract protein (5 μg) in 2.5% glycerol, 1 μg of poly(dI-dC), 50 mM KCl and 5 mM $MgCl_2$ at room temperature for 15 min. For competition assays, we added a tenfold molar excess of unlabelled oligonucleotide to the binding reaction. Samples were loaded on a 5% polyacrylamide gel. Electrophoresis was done at room temperature for 3 h at 100 V. The gel was then transferred to a nylon membrane and the biotin end-labelled DNA was detected using streptavidin-conjugated horseradish peroxidase. For supershift assay, antibody against p65 (Santa Cruz Biotech) was added to the binding reaction for 45 min on ice.

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Correspondence and requests for materials should be addressed to I.G. (igorg@steele.mgh.harvard.edu).

Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy

Hans-Guido Wendel[1], Elisa de Stanchina[1], Jordan S. Fridman[1]*, Abba Malina[2], Sagarika Ray[1], Scott Kogan[3], Carlos Cordon-Cardo[4], Jerry Pelletier[2] & Scott W. Lowe[1]

[1]*Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, USA*
[2]*McGill University, Montreal, H3G1Y6, Quebec, Canada*
[3]*University of California-San Francisco, San Francisco, California 94143-0128, USA*
[4]*Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, USA*

* Present address: Incyte Corporation, Wilmington, Delaware 19880, USA

Evading apoptosis is considered to be a hallmark of cancer, because mutations in apoptotic regulators invariably accompany tumorigenesis[1]. Many chemotherapeutic agents induce apoptosis, and so disruption of apoptosis during tumour evolution can promote drug resistance[2]. For example, Akt is an apoptotic regulator that is activated in many cancers and may promote drug resistance *in vitro*[3]. Nevertheless, how Akt disables apoptosis and its contribution to clinical drug resistance are unclear. Using a murine lymphoma model, we show that Akt promotes tumorigenesis and drug resistance by disrupting apoptosis, and that disruption of Akt signalling using the mTOR inhibitor rapamycin reverses chemoresistance in lymphomas expressing Akt, but not in those with other apoptotic defects. eIF4E, a translational regulator that acts downstream of Akt and mTOR, recapitulates Akt's action in tumorigenesis and drug resistance, but is unable to confer sensitivity to rapamycin and chemotherapy. These results establish Akt signalling through mTOR and eIF4E as an important mechanism of oncogenesis and drug resistance *in vivo*, and reveal how targeting apoptotic programmes can restore drug sensitivity in a genotype-dependent manner.

Apoptosis is controlled by a complex network of proliferation and survival genes that is frequently disrupted during tumour evolution. For example, the phosphatidylinositol-3-OH kinase (PI(3)K) pathway integrates receptor tyrosine kinase signalling with the apoptotic network[4,5]. One mediator of PI(3)K signalling is the Akt/protein kinase B (PKB) kinase, which phosphorylates multiple downstream effectors that ultimately produce global changes in cellular physiology. How Akt promotes survival is controversial, but it may involve direct phosphorylation of apoptotic regulators, increased cell cycle progression, decreased transcription of pro-apoptotic genes through inhibition of forkhead transcription factors, altered metabolism, or changes in the translation of messenger RNAs that ultimately control cell death[5].

Proliferation of cancer cells despite CDK2 inhibition

Osamu Tetsu and Frank McCormick*

Cancer Research Institute and Comprehensive Cancer Center, University of California, San Francisco, California 94143
*Correspondence: mccormick@cc.ucsf.edu

Summary

We have investigated the contribution of CDK4 and CDK2 inhibition to G1 arrest in colon cancers following inhibition of the MEK/MAP kinase pathway. CDK4 inhibition is sufficient to cause arrest, but inhibition of CDK2 by p27 Kip1 redistribution or ectopic expression has no effect on proliferation. Likewise, inhibition of CDK2 through expression of dominant-negative (DN) CDK2 or antisense oligonucleotides did not prevent cell proliferation in these cells. We therefore tested whether CDK2 activity is dispensable in other cells. Surprisingly, osteosarcomas and Rb-negative cervical cancers continued to proliferate after depletion of CDK2 through antisense oligonucleotides or small interfering (si) RNA. Here we report of sustained cell proliferation in the absence of CDK2, and we suggest that CDK2 is not a suitable target for cancer therapy.

Introduction

Genetic alterations in the Rb pathway are a hallmark of cancer and have revealed a number of possible therapeutic targets, including the cyclin-dependent kinases CDK4 and CDK2 (Sherr and Roberts, 1999; Malumbres and Barbacid, 2001). CDK4 activity is clearly implicated in cancer by alterations in its cyclin D partner and its regulator p16 Ink4a, as well as mutations in CDK4 itself (Sherr and McCormick, 2002), whereas CDK2 is not affected directly by mutations or gene copy number changes that cause cancer (Sherr and Roberts, 1999). It is currently believed that progression through the cell cycle from G1 to S phase requires sequential activation of CDK4 and CDK2 (Sherr and Roberts, 1999; Malumbres and Barbacid, 2001). The role of CDK4 is well established: it phosphorylates the Rb protein and releases E2F activity (Reed, 1997). E2F in turn activates transcription of a number of genes involved in regulating and mediating DNA synthesis (Herrera et al., 1996). CDK4 activity is dispensable in cells lacking the Rb protein: many cancer cells, for example, fail to express Rb through sporadic mutation of the Rb gene, and these cells fail to growth arrest in the presence of CDK4 inhibitors (Lukas et al., 1995a, 1995b; Koh et al., 1995; Madema et al., 1995). On the other hand, the function of CDK2 is less clear. A dominant-negative (DN) form of CDK2 prevents growth of cells in culture (van den Heuvel and Harlow, 1993; Hu et al., 2001), and microinjection of antibodies against CDK2, cyclin E, or cyclin A block initiation of DNA synthesis in mammalian cells (Ohtsubo et al., 1995; Pagano et al., 1992; Tsai et al., 1993). Furthermore, expression of the CDK2 inhibitor p27 Kip1 generally causes growth arrest (Polyak et al., 1994; Toyoshima and Hunter, 1994; Kwon and Nordin, 1997; Dirks et al., 1997; Katayose et al., 1997; Blain et al., 1997; Cheng et al., 1999; Yang et al., 2001), although exceptions have been reported (Naruse et al., 2000). However, there is little consensus on the critical substrates of CDK2 (reviewed in Reed, 1997). Rb appears to be a target (Hinds et al., 1992), though the sites of phosphorylation have been controversial (Zarkowska and Mittnacht, 1997). Other substrates for CDK2 include the centrosome protein nucleophosmin and proteins involved in DNA replication (Okuda et al., 2000; Nigg, 2001; Stucke et al., 2002). However, it has not yet been proven that phosphorylation is necessary for proliferation of mammalian cells in culture. In *Xenopus* extracts, CDK2 is necessary for sustaining multiple rounds of DNA replication, but in the initial round, other kinases such as aurora kinase may be sufficient (Nigg, 2001). However, in mammalian somatic cells, CDK2 may be regulated differently and in cancer cells, aurora kinase and related enzymes are often misregulated or overexpressed (Reed, 1997; Bischoff et al., 1998; Nigg, 2001).

In this paper, we report that CDK2 activity is dispensable for cancer cell proliferation. We will discuss the possibility that high levels of CDK4 activity in these cells may compensate for requirement of CDK2 during cell cycle progression and also suggest that in Rb-minus tumor cells, both CDK4 and CDK2 may be unnecessary for proliferation.

Results

cyclin D1, cyclin D3, CDK4, and p21 Cip1 proteins are depleted by treatment of colon carcinoma cells with MEK inhibitors, resulting in complete loss of CDK4 activity

Inhibitors of the Ras/Raf/MEK/MAP kinase pathway are currently undergoing clinical evaluation, based on their ability to

SIGNIFICANCE

This paper reports of sustained cell proliferation in the absence of CDK2 activity. Cyclin E/CDK2 activity is frequently increased in tumors, but it is not clear that this is a cause or a consequence of the disease since there is little consensus on the biochemical targets of CDK2. Rb appears to be a target, but, as shown here, CDK4 is able to phosphorylate Rb even at CDK2 preferred phosphorylation sites. We propose that increased levels of CDK4 or E2F activity in cancer cells may compensate for the requirement for CDK2 activity. These results show that CDK2 is not a suitable target for treatment of cancers, and also question the role of CDK2 in cell proliferation.

DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis

Jirina Bartkova[1], Zuzana Hořejší[1,5], Karen Koed[2], Alwin Krämer[1], Frederic Tort[1], Karsten Zieger[2], Per Guldberg[1], Maxwell Sehested[3], Jahn M. Nesland[4], Claudia Lukas[1], Torben Ørntoft[2], Jiri Lukas[1] & Jiri Bartek[1]

[1]*Institute of Cancer Biology and Centre for Genotoxic Stress Research, Danish Cancer Society, Strandboulevarden 49, DK-2100 Copenhagen, Denmark*
[2]*Department of Clinical Biochemistry, Aarhus University Hospital, Skejby, DK-8200 Aarhus N, Denmark*
[3]*Department of Pathology, University Hospital, Frederik V's Vej 11, DK-2100 Copenhagen, Denmark*
[4]*Department of Pathology, The Norwegian Radium Hospital, University of Oslo, Ullernchausseen 70-0310 Oslo, Norway*
[5]*Institute of Molecular Genetics, Czech Academy of Sciences, Flemingovo nam. 2, Praha 6, CZ-16637, Czech Republic*

During the evolution of cancer, the incipient tumour experiences 'oncogenic stress', which evokes a counter-response to eliminate such hazardous cells. However, the nature of this stress remains elusive, as does the inducible anti-cancer barrier that elicits growth arrest or cell death. Here we show that in clinical specimens from different stages of human tumours of the urinary bladder, breast, lung and colon, the early precursor lesions (but not normal tissues) commonly express markers of an activated DNA damage response. These include phosphorylated kinases ATM and Chk2, and phosphorylated histone H2AX and p53. Similar checkpoint responses were induced in cultured cells upon expression of different oncogenes that deregulate DNA replication. Together with genetic analyses, including a genome-wide assessment of allelic imbalances, our data indicate that early in tumorigenesis (before genomic instability and malignant conversion), human cells activate an ATR/ATM-regulated DNA damage response network that delays or prevents cancer. Mutations compromising this checkpoint, including defects in the ATM–Chk2–p53 pathway, might allow cell proliferation, survival, increased genomic instability and tumour progression.

Tumorigenesis is an evolutionary process that selects for genetic and epigenetic changes, allowing evasion of anti-proliferative and cell-death-inducing mechanisms that normally limit clonal expansion of somatic cells[1]. Most tumours acquire genetic instability, but how early this occurs and whether it drives tumour development is unclear[2]. Several mechanisms to constrain oncogenesis have been proposed, including hypoxia[3], telomere attrition[4] and induced expression of the Arf tumour suppressor (which is caused by the mitogenic overload experienced by incipient cancer cells)[5]. These are all conditions that can activate the tumour suppressor p53 (refs 1, 3–6). However, whether these mechanisms represent the major force(s) that guard against genetic instability and tumorigenesis is unknown. Recently, another possibility emerged from our observation[7] that advanced carcinomas of the lung and breast show constitutive activation of Chk2, an effector kinase[8] within the DNA damage network that is activated by the kinase ATM (Ataxia Telangiectasia Mutated) in response to DNA double-strand breaks[9,10]. Furthermore, oncogenes such as Myc cause DNA damage in cultured cells[11,12]. These findings led us to hypothesize that DNA damage checkpoints might become activated in the early stages of human tumorigenesis, leading to cell-cycle blockade or apoptosis and thereby constraining tumour progression.

DNA damage signalling in early bladder tumours

To determine whether Chk2 is activated in premalignant human tumours, we compared early, superficial lesions (stage Ta), early invasive (T1) and more advanced stages (T2–4) of urinary bladder cancer, all untreated by radiation or chemotherapy. Contrary to the negative staining of normal tissues, immunohistochemistry using a well-characterized antibody[7,8] against activated Chk2 (phosphorylated at Thr 68) showed heterogeneous positive staining in the vast majority of the Ta lesions (Figs 1 and 2a). A similar pattern was seen in the T1 tumours, but the T2–4 carcinomas, although still commonly positive, showed moderately lower staining (Figs 1 and 2a).

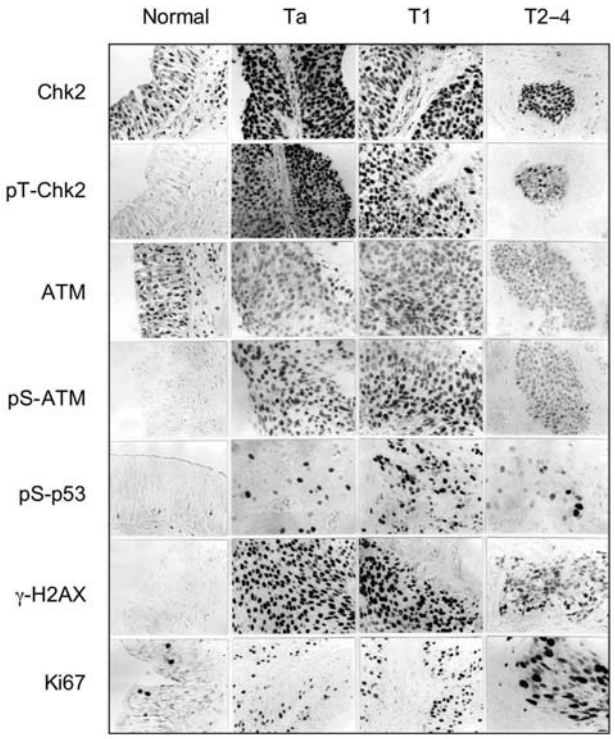

Figure 1 Constitutive activation of the ATM–Chk2–p53 pathway in human urinary bladder cancer. Immunohistochemistry of normal uroepithelium, early superficial lesions (Ta), earliest invasive (T1) and more advanced primary carcinomas (T2–4). Chk2 and ATM proteins are ubiquitously expressed, but Thr 68-phosphorylated Chk2 (pT-Chk2), Ser 1981-phosphorylated ATM (pS-ATM), Ser 15-phosphorylated p53 (pS-p53) and Ser 139-phosphorylated histone H2AX (γ-H2AX) are detectable only in tumour tissues. They are all present at the early stages of tumour development. Ki67 is a marker of proliferating cells. Original magnification, ×100.

Aborting the birth of cancer

Ashok R. Venkitaraman

Can cells sense and stop uncontrolled division driven by cancer-promoting stimuli? Perhaps so, given evidence that aberrant division can trigger the cellular response to DNA damage — blocking growth — at early stages in human cancer.

Why human cancer is not more frequent remains a mystery, given our trillions of susceptible cells, each with many genes subject to mutations that could ignite uncontrolled cell proliferation. One intuitive concept — which has been in the spotlight for decades — is that normal cells can somehow perceive and arrest aberrant cycles of cell division that are triggered by cancer-promoting (oncogenic) stimuli, such as the inappropriate activation of oncogenes. But how cells might do so remains elusive.

On pages 864 and 907 of this issue, Bartkova *et al.*[1] and Gorgoulis *et al.*[2] supply evidence that oncogene-driven cell-division cycles trigger DNA damage associated with DNA replication (the process that faithfully copies the genome in preparation for division). This DNA damage raises a barrier to sustained proliferation. From these findings, a fresh picture emerges, in which progression towards full-blown cancer requires the wayward cell to inactivate the mechanisms that monitor damage during DNA replication. This would help to explain the close link between genomic instability and cancer evolution, and extend our theoretical framework for understanding how cancers develop.

Early clues to the existence of mechanisms that prevent uncontrolled cell division came from the observation, more than 20 years ago, that viral oncogenes arrest the proliferation of normal cells in culture[3,4]. Later, the tumour-suppressor proteins p53 and ARF were found to be vital for constraining oncogene-driven proliferation[5,6]. Their activation was variously attributed to excessive stimulation to proliferate, oxidative stress, or the loss of appropriate signals from the tissue microenvironment[7] — all triggered by oncogenic stimuli. Activation of these tumour suppressors causes cells either to become dormant (senesce) or to commit suicide (by the process of 'apoptosis'). But evidence that these constraints on proliferation operate during human cancer development has been hard to find.

Enter Bartkova, Gorgoulis and their colleagues[1,2], who propose from studies of human cancer samples that another constraint limits aberrant cell division. They provide evidence that the cellular response to DNA damage — specifically, to double-strand breaks in DNA — is activated in early

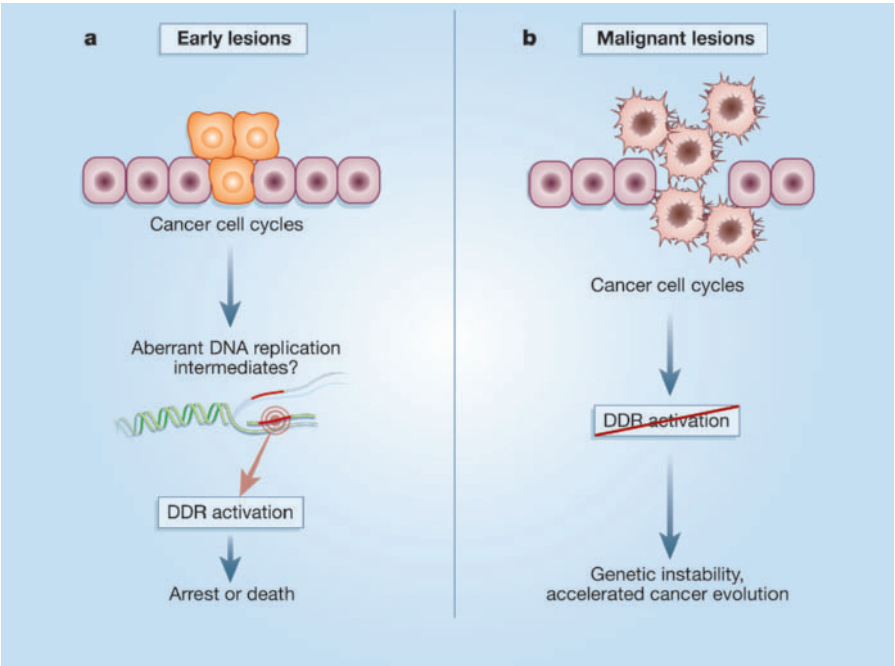

Figure 1 **Sensing and stopping wayward cell divisions.** a, **Bartkova *et al.*[1] and Gorgoulis *et al.*[2] provide evidence that, in early cancerous lesions, cell-division cycles driven by oncogenic stimuli ('cancer cell cycles') trigger the cellular DNA-damage response (DDR), as a result of aberrations in DNA replication. The nature of these aberrations is uncertain. The DDR then arrests cell proliferation or causes cell death. This might create a selection pressure for suppression of the DDR during carcinogenesis.** b, **Hence, the progression to malignant lesions might be accompanied by DDR inactivation, which would in turn create genetic instability and accelerate cancer evolution. What distinguishes cancer cell cycles from normal division at the level of DNA replication remains a key, unresolved question.**

lesions from lung or bladder tumours. This evidence includes the presence of active forms of ATM or Chk2, participants in the enzymatic cascade that responds to double-strand breaks[8]. Notably, these markers are detected in precancerous lesions — where there is evidence for oncogene-induced aberrant division, but not yet for the changes typical of full-blown cancers — suggesting that the DNA-damage response (DDR) is activated at the earliest stages in carcinogenesis (Fig. 1a). Moreover, the markers are absent from normal proliferating epithelial cells, and from inflammatory lesions, indicating that they discriminate normal from aberrant cell cycles.

To verify and extend these observations, the authors either overexpress oncogenes such as the cell-cycle regulator cyclin E in tissue-culture cells[1], or graft human skin sections onto the backs of immunodeficient mice and use growth factors to induce hyperproliferation of skin cells[2]. In both cases, the abnormal cell cycles elicit the DDR *in vitro*. This also occurs after inactivation of the tumour-suppressor protein Rb, which ordinarily serves as a gatekeeper for entry into the cell cycle — suggesting that the DDR can be initiated by numerous alterations that underlie the uncontrolled division of cancer cells.

The DDR arrests cell division, and can trigger apoptosis[9]. The authors propose[1,2] that the need for cells to surmount this barrier during carcinogenesis creates a selection pressure for the inactivation of p53 or other participants in the DDR (Fig. 1b). This, in turn, causes genetic instability — increasing the mutation rate, and accelerating cancer evolution. From this perspective, genetic instability is an unavoidable by-product of the breakdown of barriers to uncontrolled division during early stages of carcinogenesis.

This view raises several questions, the most important of which is how the abnormal

with biotinylated horse anti-mouse IgG and the ABC-Elite reagent. In all cases 3,3′-diaminobenzidine (with nickel chloride enhancement for SP-C and CC10 staining) was used as the chromagen and sections were counterstained with methyl green.

Analysis of Env RNA expression in lung and airways

Total RNA was isolated from lung, from trachea and from epithelial tissue scraped from the inside of the nose, by using a Polytron tissue homogenizer (Brinkmann) and Trizol RNA isolation reagent (Invitrogen). Samples were treated with DNase and with reverse transcriptase in the presence of a 3′ Env primer; they were then subjected to 30 cycles of PCR amplification with primers flanking the intron in ARJenv (Fig. 1). Products were subjected to electrophoresis in agarose gels and were stained with ethidium bromide.

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Correspondence and requests for materials should be addressed to A.D.M. (dmiller@fhcrc.org).

Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions

Vassilis G. Gorgoulis[1]*, Leandros-Vassilios F. Vassiliou[1]*, Panagiotis Karakaidos[1], Panayotis Zacharatos[1], Athanassios Kotsinas[1], Triantafillos Liloglou[2], Monica Venere[3,4], Richard A. DiTullio Jr[3,4], Nikolaos G. Kastrinakis[1], Brynn Levy[6], Dimitris Kletsas[7], Akihiro Yoneta[3], Meenhard Herlyn[3], Christos Kittas[1] & Thanos D. Halazonetis[3,5]

[1]*Department of Histology and Embryology, School of Medicine, University of Athens, Athens GR-11527, Greece*
[2]*Roy Castle Lung Cancer Research Programme, Cancer Research Center, University of Liverpool, Liverpool L3 9TA, UK*
[3]*The Wistar Institute, Philadelphia, Pennsylvania 19104-4268, USA*
[4]*Graduate Group in Biomedical Sciences and*
[5]*Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA*
[6]*Department of Human Genetics, Mount Sinai School of Medicine, New York, New York 10029, USA*
[7]*Institute of Biology, National Centre of Scientific Research 'Demokritos', Athens GR-15310, Greece*

* These authors contributed equally to this work

DNA damage checkpoint genes, such as *p53*, are frequently mutated in human cancer, but the selective pressure for their inactivation remains elusive[1–3]. We analysed a panel of human lung hyperplasias, all of which retained wild-type *p53* genes and had no signs of gross chromosomal instability, and found signs of a DNA damage response, including histone H2AX and Chk2 phosphorylation, p53 accumulation, focal staining of p53 binding protein 1 (53BP1) and apoptosis. Progression to carcinoma was associated with *p53* or *53BP1* inactivation and decreased apoptosis. A DNA damage response was also observed in dysplastic nevi and in human skin xenografts, in which hyperplasia was induced by overexpression of growth factors. Both lung and experimentally-induced skin hyperplasias showed allelic imbalance at loci that are prone to DNA double-strand break formation when DNA replication is compromised (common fragile sites). We propose that, from its earliest stages, cancer development is associated with DNA replication stress, which leads to DNA double-strand breaks, genomic instability and selective pressure for *p53* mutations.

The most frequently mutated gene in human cancer is *p53*, a gene that functions in the checkpoint response to DNA double-strand breaks (DSBs; Fig. 1a)[1,2]. Several models (not mutually exclusive) have been proposed to explain the high frequency of *p53* inactivation[3–6]. One of the prevailing models states that tumour growth leads to telomere attrition and hypoxia, resulting in a DNA damage response[4–6]. This model predicts that the DNA damage response occurs some time after cancer initiation[3]. Here, we performed a systematic analysis of precancerous and cancer lesions to determine how early during human cancer development a DNA DSB checkpoint response might become apparent.

We first examined a previously described panel of surgically-resected, non-small cell lung carcinomas (NSCLCs) from patients who had received no form of cancer therapy before surgery[7,8]. Almost all specimens in this panel ($n = 74$) contained normal adjacent lung tissue ($n = 72$), and some also contained hyperplastic ($n = 17$) and dysplastic lesions ($n = 2$), the location of which suggested that they were precursors of the NSCLCs. The *p53* gene was wild-type in all of the hyperplasias, mutant in the dysplasias, and either mutant ($n = 45$) or wild-type ($n = 29$) in the NSCLCs. For the two dysplasias, the same *p53* mutations were found in the adjacent NSCLCs, consistent with the dysplasias being precursors of the adjacent NSCLCs (data not shown).

REVIEW

Shelterin: the protein complex that shapes and safeguards human telomeres

Titia de Lange[1]

The Rockefeller University, New York, New York 10021, USA

Added by telomerase, arrays of TTAGGG repeats specify the ends of human chromosomes. A complex formed by six telomere-specific proteins associates with this sequence and protects chromosome ends. By analogy to other chromosomal protein complexes such as condensin and cohesin, I will refer to this complex as shelterin. Three shelterin subunits, TRF1, TRF2, and POT1 directly recognize TTAGGG repeats. They are interconnected by three additional shelterin proteins, TIN2, TPP1, and Rap1, forming a complex that allows cells to distinguish telomeres from sites of DNA damage. Without the protective activity of shelterin, telomeres are no longer hidden from the DNA damage surveillance and chromosome ends are inappropriately processed by DNA repair pathways. How does shelterin avert these events? The current data argue that shelterin is not a static structural component of the telomere. Instead, shelterin is emerging as a protein complex with DNA remodeling activity that acts together with several associated DNA repair factors to change the structure of the telomeric DNA, thereby protecting chromosome ends.

Six shelterin subunits: TRF1, TRF2, TIN2, Rap1, TPP1, and POT1

The components of shelterin were gradually identified over the past 10 years (Fig. 1). The first mammalian telomeric protein, now referred to as TRF1, was isolated based on its in vitro specificity for double-stranded TTAGGG repeats typical of vertebrate telomeres (Zhong et al. 1992; Chong et al. 1995). TRF2 was identified as a TRF1 paralog in the database (Bilaud et al. 1997; Broccoli et al. 1997) and TIN2 and Rap1 were found in two-hybrid screens with TRF1 and TRF2, respectively (Kim et al. 1999; Li et al. 2000). TPP1 (previously called TINT1 [Houghtaling et al. 2004], PTOP [Liu et al. 2004b], and PIP1 [Ye et al. 2004b]) recently emerged from searches for TIN2-interacting proteins. The most conserved component of shelterin, POT1, was identified based on sequence homology to telomere end-binding factors in unicellular eukaryotes (Baumann and Cech 2001). Mass spectrometry on shelterin-associated factors failed to deliver additional components, suggesting that the tally of its subunits is nearing completion (Liu et al. 2004b; O'Connor et al. 2004; Ye et al. 2004a).

All six shelterin subunits can be found in a single complex in fractionated nuclear extracts (Liu et al. 2004a; Ye et al. 2004a). The linchpin of shelterin is TIN2, which tethers TPP1/POT1 to TRF1 and TRF2. TIN2 also connects TRF1 to TRF2 and this link contributes to the stabilization of TRF2 on telomeres (Liu et al. 2004a; Ye et al. 2004a). Shelterin subcomplexes containing either TRF1 or TRF2 in association with the other subunits can also be isolated. Although these subcomplexes could be an isolation artifact of the salt sensitivity of the TIN2–TRF2 link (Ye et al. 2004a), photobleaching experiments also suggest that some of TRF1 and TRF2 are in separate complexes (Mattern et al. 2004). Further work is needed to establish the number of shelterin units bound per telomere, the stoichiometry of the shelterin subunits, and the significance of shelterin subcomplexes.

Not all proteins at chromosome ends are part of shelterin. Several criteria distinguish the shelterin components from the non-shelterin proteins observed at telomeres (Table 1). Shelterin is abundant at chromosome ends but does not accumulate elsewhere; it is present at telomeres throughout the cell cycle, and its known function is limited to telomeres. Non-shelterin proteins at chromosome ends fail to meet two or three of these criteria, yet can play important roles at telomeres.

Shelterin has exquisite specificity for telomeric TTAGGG repeats due to the presence of multiple TTAGGG recognition folds in the complex. The SANT/Myb-type DNA-binding domains of TRF1 and TRF2 each bind the sequence 5′-YTAGGGTTR-3′ in duplex DNA, showing very low tolerance for single-base changes (Fig. 1; Bianchi et al. 1999; Court et al. 2005; Hanaoka et al. 2005). TRF1 and TRF2 each form homodimers and higher order oligomers, so the collection of multiple DBDs they bring to the complex can peruse a large DNA sequence. POT1 also has strong sequence specificity, binding single-stranded 5′-(T)TAGGGT TAG-3′ sites both at a 3′ end and at internal positions (Fig. 1; Lei et al. 2004; Loayza et al. 2004). Since these three shelterin subunits are connected through protein–

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[1]**Correspondence.**
E-MAIL delange@mail.rockefeller.edu; FAX (212) 327-7147.

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MicroRNAs: SMALL RNAs WITH A BIG ROLE IN GENE REGULATION

Lin He and Gregory J. Hannon

MicroRNAs are a family of small, non-coding RNAs that regulate gene expression in a sequence-specific manner. The two founding members of the microRNA family were originally identified in *Caenorhabditis elegans* as genes that were required for the timed regulation of developmental events. Since then, hundreds of microRNAs have been identified in almost all metazoan genomes, including worms, flies, plants and mammals. MicroRNAs have diverse expression patterns and might regulate various developmental and physiological processes. Their discovery adds a new dimension to our understanding of complex gene regulatory networks.

RNA INTERFERENCE (RNAi). A form of post-transcriptional gene silencing, in which dsRNA induces degradation of the homologous mRNA, mimicking the effect of the reduction, or loss, of gene activity.

Cold Spring Harbor Laboratory, Watson School of Biological Sciences, 1 Bungtown Road, Cold Spring Harbor, New York 11724, USA. Correspondence to G.J.H. e-mail: hannon@cshl.edu

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Non-coding RNAs participate in a surprisingly diverse collection of regulatory events, ranging from copy-number control in bacteria[1] to X-chromosome inactivation in mammals[2]. MicroRNAs (miRNAs) are a family of 21–25-nucleotide small RNAs that, at least for those few that have characterized targets, negatively regulate gene expression at the post-transcriptional level[3–5]. Members of the miRNA family were initially discovered as small temporal RNAs (stRNAs) that regulate developmental transitions in *Caenorhabditis elegans*[6]. Over the past few years, it has become clear that stRNAs were the prototypes of a large family of small RNAs, miRNAs, that now claim hundreds of members in worms, flies, plants and mammals. The functions of miRNAs are not limited to the regulation of developmentally timed events. Instead, they have diverse expression patterns and probably regulate many aspects of development and physiology[3,4,7–9]. Although the mechanisms through which miRNAs regulate their target genes are largely unknown, the finding that at least some miRNAs feed into the RNA INTERFERENCE (RNAi) pathway has provided a starting point in our journey to understand the biological roles of miRNAs.

In this review, we revisit the history of miRNAs and summarize recent findings in miRNA biogenesis, translational repression and biological function. We conclude by highlighting the continuing genome-wide efforts to identify novel miRNAs and to predict their targets.

The discovery of miRNAs

The founding member of the miRNA family, *lin-4*, was identified in *C. elegans* through a genetic screen for defects in the temporal control of post-embryonic development[10,11]. In *C. elegans*, cell lineages have distinct characteristics during 4 different larval stages (L1–L4). Mutations in *lin-4* disrupt the temporal regulation of larval development, causing L1 (the first larval stage)-specific cell-division patterns to reiterate at later developmental stages[10]. Opposite developmental phenotypes — omission of the L1 cell fates and premature development into the L2 stage — are observed in worms that are deficient for *lin-14* (REF. 12). Even before the molecular identification of *lin-4* and *lin-14*, these loci were placed in the same regulatory pathway on the basis of their opposing phenotypes and antagonistic genetic interactions[11]. Most genes identified from mutagenesis screens are protein-coding, but *lin-4* encodes a 22-nucleotide non-coding RNA that is partially complementary to 7 conserved sites located in the 3′-untranslated region (UTR) of the *lin-14* gene (FIG. 1b)[13,14]. *lin-14* encodes a nuclear protein, downregulation of which at the end of the first larval stage initiates the developmental progression into the second larval stage[13,15]. The negative regulation of LIN-14 protein expression requires an intact 3′ UTR of its mRNA[14], as well as a functional *lin-4* gene[13]. These genetic interactions inspired a series of molecular and biochemical studies demonstrating that

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Mediator special issue

Mediator and the mechanism of transcriptional activation

Roger D. Kornberg

Department of Structural Biology, Stanford School of Medicine, Stanford, CA 94305, USA

Mediator was discovered because of its activity in a yeast RNA polymerase II (pol II) transcription system – it is needed for the system to respond to a transcriptional activator. Mediator is the central link in the enhancer→activator→Mediator→pol II→promoter pathway. The transduction of regulatory signals through this pathway is crucial for transcription of almost all pol II promoters in all eukaryote organisms.

Introduction

The ultimate goal of research on transcription is an understanding of transcriptional control. In the case of bacteria and bacteriophage, this goal has been largely achieved. Transcriptional repressor and activator proteins, which are responsive to environmental stimuli, bind to DNA sequences that are adjacent to promoters and exert effects directly on RNA polymerase. Repressors prevent polymerase binding to the promoter, whereas activators contact polymerase and increase its affinity for the promoter or stimulate the transition from a closed to an open polymerase–promoter complex (formation of a 'transcription bubble', in which the DNA double helix is melted to facilitate the initiation of transcription).

At one level, a similar basis was found for control of transcription in eukaryotes. A signal of intracellular or environmental origin affects the state of a regulatory protein – its nuclear localization, its half-life, or its activity – with a consequent effect on transcription. At another level, the problem remained: how are multiple regulatory signals, which impinge on complex eukaryotic promoters, processed and transmitted to RNA polymerase II (pol II)? The solution of this problem has been found in features of the transcription machinery that are unique to eukaryotic cells.

The central components of the transcription machinery are the same in bacteria and eukaryotic cells. The RNA polymerases share a conserved core and common transcription mechanism. The initiation factors – σ in bacteria and a set of general transcription factors (GTFs) in eukaryotes – are more distantly related, but function in a similar manner in promoter recognition, promoter melting, abortive initiation and promoter escape. Where bacterial and eukaryotic systems truly diverge is in the targets of regulatory proteins. In contrast with the direct targeting of RNA polymerase in bacteria, there are intermediary factors in eukaryotes: chromatin and Mediator. Chromatin of eukaryotes, which is based on a histone octamer enveloped by DNA, and Mediator, a giant multiprotein complex, have no counterparts in bacteria. They represent a new layer interposed between the regulatory proteins and RNA polymerase. This layer must account for the greater complexity of regulation in eukaryotes and the consequent capacity for cell differentiation and development.

Discovery of Mediator

Although ostensibly an exercise of biochemistry, involving the fractionation of a yeast extract, the isolation of Mediator was anything but straightforward. The reason for this was to do with the complexity of the transcription system, the vagaries of the protein factors and the very definition of transcriptional activation. The yeast system was advantageous for the research: (i) it provided an early indication of the existence of Mediator and, thus, motivation for persisting despite the difficulties; and (ii) it provided validation of the final result, establishing both the physiological relevance and the broader implications of Mediator for control of transcription.

The earliest evidence for Mediator came from biochemical studies in yeast. It was previously shown that overexpression in yeast of one activator interferes with the activation of pol II transcription by another [1]. This effect, termed 'squelching', was attributed to competition between activators for a common target that was present in a limiting amount in yeast. The target was believed to be a component of the pol II transcription machinery – either one of the GTFs or pol II itself. This idea was proved by the demonstration of activator binding to the TATA-binding protein (TBP) subunit of the GTF TFIID [2]. Activators were subsequently shown to bind to TFIIB, TFIIH and pol II [3–5]. The promiscuity of activator interactions did not shake confidence in a direct mechanism of transcriptional activation. However, the widespread belief in a direct mechanism was finally challenged by analysis of squelching in a crude yeast pol II transcription system *in vitro* [6]. On the one hand, addition of an excess of any of the GTFs or of pol II failed to relieve squelching, which argued against these proteins being the activator target. On the other hand, addition of a crude protein fraction from yeast did relieve squelching, and the activity of this crude fraction was termed Mediator.

For isolation of Mediator from the crude yeast fraction, a better assay than the relief of squelching was required.

Corresponding author: Kornberg, R.D. (kornberg@stanford.edu).
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Mediator special issue

The mammalian Mediator complex and its role in transcriptional regulation

Ronald C. Conaway[1,2], Shigeo Sato[1], Chieri Tomomori-Sato[1], Tingting Yao[1] and Joan W. Conaway[1,2,3]

[1]Stowers Institute for Medical Research, Kansas City, MO 64110, USA
[2]Department of Biochemistry and Molecular Biology, Kansas University Medical Center, Kansas City, KS 66160, USA
[3]Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190, USA

Mediator is an essential component of the RNA polymerase II general transcriptional machinery and plays a crucial part in the activation and repression of eukaryotic mRNA synthesis. The *Saccharomyces cerevisiae* Mediator was the first to be defined and is a high molecular mass complex composed of >20 distinct subunits that performs multiple activities in transcription. Recent studies have defined the subunit composition and associated activities of mammalian Mediator, and revealed a striking evolutionary conservation of Mediator structure and function from yeast to man.

Introduction

The initiation stage of mRNA synthesis is a major site for the regulation of gene expression. In eukaryotes, mRNA synthesis is catalyzed by the multisubunit enzyme RNA polymerase II (pol II) and regulated by a host of DNA-binding transcription factors that activate or repress transcription in response to a myriad of signals emanating both from within the cell and from the cellular environment. Biochemical studies have shown that the initiation and regulation of eukaryotic mRNA synthesis requires a large collection of evolutionarily conserved 'general' transcription factors that seem to function at all, or most, genes. These general transcription factors include (i) the general initiation factors TFIIB, TFIID, TFIIE, TFIIF and TFIIH, which constitute the minimal set of auxiliary proteins necessary and sufficient for selective binding and accurate transcription initiation *in vitro* by pol II from the core regions of most promoters [1–4]; and (ii) the multiprotein Mediator complex, which functions, at least in part, as an adaptor that supports essential communication from transcription factors bound at upstream promoter elements and enhancers to pol II and the general initiation factors at the core promoter [5,6].

Although the compositions and many of the functions of the general initiation factors from yeast and higher eukaryotes were well established by the early 1990s, the structure and activities of the Mediator complex have only recently been illuminated. Mediator was first discovered and purified to near homogeneity from *Saccharomyces cerevisiae* by Kornberg and coworkers. They showed that it is required for transcription activation by the transcriptional activators Gcn4 or GAL4-VP16 *in vitro* using a reconstituted enzyme system composed of purified pol II and general initiation factors [7–9]. Yeast Mediator is composed of ~20 subunits, which are present in three distinct Mediator subdomains referred to as the 'head,' 'middle' and 'tail' modules (reviewed in Ref. [10]) (Figure 1). An additional module, which includes a kinase–cyclin pair, is associated with a subset of yeast Mediator complexes and has, in yeast that is growing exponentially, been implicated in repression of a subset of genes [11–14].

Mammalian Mediator-like complexes were subsequently identified and characterized in several laboratories. However, whether the mammalian Mediator-like complexes isolated in different laboratories represented the same or different functional entities was unclear at first because they seemed to include distinct, but overlapping, sets of subunits. Furthermore, there was considerable controversy over the evolutionary relationship between yeast Mediator and mammalian Mediator-like complexes because there were obvious mammalian orthologs of only a subset of yeast Mediator subunits. As described in more detail later, recent efforts exploiting state-of-the-art proteomics methods have defined a set of consensus mammalian Mediator subunits, and improved bioinformatics approaches have revealed a striking evolutionary conservation of Mediator from yeast to man.

Here, we discuss these recent developments in studies of the structure and function of mammalian Mediator.

Isolation of mammalian Mediator-like complexes

Mammalian Mediator-like complexes have been isolated by a variety of methods, including conventional and affinity chromatography. The first such complex was purified by Roeder and coworkers. It was designated the TRAP (thyroid hormone receptor-associated proteins) complex because it was isolated in association with the liganded thyroid hormone receptor (TR) [15,16]. Subsequently, related Mediator-like complexes were purified in several laboratories by conventional chromatography from mouse B cells [17], human HeLa cells [18–20], and rat liver [21] and designated mouse Mediator, CRSP

Corresponding author: Conaway, J.W. (jlc@stowers-institute.org).
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PROGRESS

Reversing histone methylation

Andrew J. Bannister[1] & Tony Kouzarides[1]

Histones package DNA, and post-translational modifications of histones can regulate access to DNA. Until recently, histone methylation—unlike all other histone modifications—was considered a permanent mark. The discovery of enzymes that reverse the methylation of lysines and arginines challenges our current thinking on the unique nature of histone methylation, and substantially increases the complexity of histone modification pathways.

In its 'naked' form, DNA is unwieldy and unmanageable for a cell to package. This problem is solved by histones, which compact and control DNA. The many different types of histone modifications (for example, acetylation, methylation, phosphorylation and ubiquitination; reviewed in refs 1–3) regulate DNA-based events in ways that were unimaginable a decade ago. Histone methylation, perhaps more than any other form of modification, has demonstrated the power of modifications over DNA-based functions, regulating fundamental processes such as gene transcription and DNA repair. Furthermore, since the discovery of the first histone methyltransferase[4], the potential for the methylation 'mark' to control epigenetic events has caught the imagination of workers in this field. However, the recent discovery that methylation can be reversed[5–7] has shaken the dogma that a 'permanent' methylation mark is necessary for epigenetic control.

Histones may be methylated on either lysine (K) or arginine (R) residues. It is possible that methylation induces alterations in chromatin architecture, either condensing or relaxing its structure. However, a methyl group is relatively small and its addition to lysine or arginine residues does not neutralize their charge, so it is unlikely that methylation alone will significantly affect chromatin structure. It is more likely that it creates binding sites for regulatory proteins that contain specialized binding domains.

Lysine side chains may be mono-, di- or tri-methylated, whereas the arginine side chain may be mono-methylated or (symmetrically or asymmetrically) di-methylated[3,8]. At present, there are 24 known sites of methylation on histones (17 are lysine residues and 7 are arginine residues). If we take into consideration all three possible methylation states of lysine and arginine, there are potentially 3×10^{11} distinct methylation states of histone proteins. Although all of this combinatorial specificity may not be used, this calculation highlights the vast potential for the regulation of function, and the enormity of the task of understanding how methylation works. Why are there such a huge number of possibilities? Are there specific functions that are controlled by a subset of modifications? Is this combinatorial specificity predictive, like a code? How do specific modifications give rise to appropriate biological outcomes? Here we review what is currently known about methylation and its control of chromatin function, and consider the implications of recent reports indicating that the methylation of histones is a dynamic process.

Methylation of lysines

The most-studied sites of lysine methylation lie in the amino termini of H3 and H4 histone proteins (Table 1). At a first level of characterization, these methylated sites are defined by their presence within a certain type of chromatin, either heterochromatin (a condensed and 'transcriptionally silent' chromatin) or euchromatin (a loosely packed and 'transcriptionally active' chromatin). In certain cases, the enzymes that mediate the methylation have been shown to direct the formation of specific chromatin states and to be responsible for transcriptional regulation (Table 1).

It is becoming clear that not all heterochromatin is the same with respect to the methylated histones that it contains. The methylated sites on the histones found within heterochromatin (H3K9, H3K27, H3K79 and H4K20) demarcate subdomains; tri-methylated H3K9 and tri-methylated H4K20 are enriched in pericentric heterochromatin, whereas tri-methylated H3K27 is enriched at the inactive X-chromosome[9–15]. This information could imply the existence of some sort of code, but whether this is predictive, with respect to the chromatin structure formed at these sites, remains to be established.

As with heterochromatin, not all euchromatin is the same. Genes within euchromatin have the potential to be active and are associated with methylated H3K4 and H3K36 histones. When a gene is expressed in yeast, further rounds of histone methylation appear in a localized fashion (enriched at the 5′ end of the gene) and in specific forms, primarily tri-methylation (reviewed in ref. 3). A large-scale analysis of human euchromatin indicates that a situation similar to the one in yeast may also occur in mammals[16].

The extent of our knowledge regarding the mechanistic and functional consequences of methylation is limited to the proteins and domains that recognize the modification. Repressive proteins, such as heterochromatin protein 1 (HP1) or the *Drosophila* Polycomb (PC) protein, contain a chromodomain that allows them to specifically recognize the appropriate repressive methylation mark (H3K9 and H3K27 respectively; reviewed in ref. 3), whereas the chromodomain helicase DNA-binding protein 1 (CHD1) activator protein from *Saccharomyces cerevisiae* uses its chromodomain to bind the activating methylated H3K4 (ref. 17). Therefore, the ultimate function of the methyl group is a reflection of the type of protein it has evolved to recruit—either an activator or a repressor of transcription (Fig. 1).

Recently, two domains that are distinct from the chromodomain were shown to bind methylated lysine residues. The Tudor domain within the DNA-repair checkpoint protein p53-binding protein 1 (p53BP1) recognizes methylated H3K79, a widely distributed histone modification in mammalian cells[18]. This finding fulfils the prediction for members of the larger Royal Family domain, which were thought to bind methylated lysine[19]. The WD40 repeats of the vertebrate transcriptional activator WDR5 also forms a binding site for a methylated lysine, in this case di- and tri-methylated H3K4 (ref. 20). The challenge in the future is to understand how the recruitment of specific proteins to methylated sites mediates the desired biological function.

Almost all methylation marks characterized to date have been shown to have a role in transcription. This monopoly of function is likely to be less a reflection of a unique role for methylation than of a bias in the current research. There is no reason to believe that other DNA functions, such as replication, recombination and repair, are

[1]Gurdon Institute and Department of Pathology, Tennis Court Road, Cambridge CB2 1QN, UK.

ELSEVIER

The key to development: interpreting the histone code?

Raphael Margueron*, Patrick Trojer* and Danny Reinberg

Developmental stages in multicellular organisms proceed according to a temporally and spatially precise pattern of gene expression. It has become evident that changes within the chromatin structure brought about by covalent modifications of histones are of crucial importance in determining many biological processes, including development. Numerous studies have provided evidence that the enzymes responsible for the modifications of histones function in a coordinated pattern to control gene expression in the short term and, through the transferral of these modifications by inheritance to their progeny, in the long term.

Addresses
Howard Hughes Medical Institute, Division of Nucleic Acids Enzymology, Department of Biochemistry, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, 683 Hoes Lane, Piscataway, NJ 08854, USA
* R Margueron and P Trojer contributed equally to this review.

Corresponding author: Reinberg, Danny (reinbedf@umdnj.edu)

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Introduction

Understanding the molecular pathways that govern development at the transcriptional level in higher eukaryotes has been an actively pursued and long awaited goal particularly as this knowledge might be crafted into well-designed approaches to tackle disease. With the current milestones achieved in clarifying the pivotal role of histone modifications in programing DNA for transcriptional regulation, the long awaited goal now appears tenable. This review surveys the current status of histone modifications, how they come about, their putative coding capacity and their role in development.

Eukaryotic genomic DNA in the nucleus, with a diameter of up to 10 microns, is compacted more than 10 000-fold by highly basic proteins known as histones. The result is a highly structured entity termed chromatin. The fundamental unit of chromatin, the nucleosome core particle, consists of 147 bp of super helical DNA wrapped in 1.75 turns around a histone octamer core. A centrally located histone $(H3/H4)_2$ tetramer is assembled with two histone H2A/H2B dimers [1]. Consecutive nucleosomes line up, generating a fiber with a diameter of 11 nm, termed beads-on-a-string, which can be further compacted into a 30 nm fiber at least partially through incorporation of the linker histone H1 [2,3]. The processes responsible for this higher order architecture are still not fully understood. Over the last two decades it has become evident that chromatin is a highly flexible environment, wherein spatially and temporally coordinated changes between transcriptionally repressive/structurally condensed states, and transcriptionally active/structurally accessible states regulate gene expression.

Initially, histones were regarded as merely structural components but now are recognized for their important role in maintaining the dynamic equilibrium of chromatin through which the regulation of gene expression is attained throughout all stages of the development of multicellular organisms. The amino termini of histones (histone tails) are accessible, unstructured domains that protrude out of the nucleosomes. Histones, especially residues of the amino termini of histones H3 and H4 and the amino and carboxyl termini of histones H2A, H2B and H1, are susceptible to a variety of post-translational modifications (Figure 1): phosphorylation (of S and T residues) [4]; acetylation (K) [5,6]; methylation (K and R) [7]; ubiquitination (K) [8]; sumoylation (K) [9]; ADP ribosylation [10]; glycosylation [11]; biotinylation [12], and carbonylation [13]. Although the first three types of modifications have been studied extensively [14], relatively little is known about the others.

Histone methylation is catalyzed by histone methyltransferases (HMTs) and is considerably different from the other types of modifications. First, histone lysine methylation appears to be irreversible, at least thus far, as histone demethylases have yet to be discovered (see Update). Because of this stability, methyl marks provide an excellent epigenetic mechanism for the stable transfer of gene expression profiles to progeny cells. Second, HMTs can be grouped into two divergent families: histone lysine methyltransferases (HKMTs) catalyzing the methylation of lysine residues (for review, see [15–17]) and protein arginine methyltransferases (PRMTs) [7] catalyzing the methylation of arginine residues. (Figure 1 represents an updated list of the mammalian HMTs with their target residues.) Third, histone methylation marks exhibit disparate outcomes with respect to gene expression involving activation and repression. This contrasts with acetylation/deacetylation of the histone

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DNA methylation and histone modifications: teaming up to silence genes

François Fuks

DNA methylation, histone deacetylation, and methylation of histone H3 at lysine 9 are the three best-characterized covalent modifications associated with a repressed chromatin state. Recent advances highlight an essential, intricate web of interactions among these processes, generating a self-reinforcing, self-perpetuating cycle of epigenetic events that lead to long-term transcriptional repression. Histone deacetylation and methylation at lysine 9 of H3 might also contribute to the establishment of DNA methylation patterns, a long-standing mystery in epigenetics. What's more, recent clues suggest a potential link between CpG methylation and other histone modifications. A complex picture is emerging in which DNA methylation and histone modifications work hand-in-hand as parts of an epigenetic program that integrates gene-silencing networks within the cell.

Addresses
Free University of Brussels, Faculty of Medicine, Laboratory of Molecular Virology, 808 route de Lennik, 1070 Brussels, Belgium

Corresponding author: Fuks, François (ffuks@ulb.ac.be)

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Introduction

DNA methylation, chromatin structure, and gene silencing are interconnected in mammals. This has been known for many years. Early studies revealed, for example, that high levels of CpG methylation coincide with heterochromatic regions. Also, upon integration into the genome, *in vitro* methylated DNA was shown to associate with a repressed chromatin structure. Other work revealed that unmethylated CpG-island chromatin is enriched in hyperacetylated histones [1]. The mechanisms underlying these observations have long remained obscure, but a recent boom of new findings on how chromatin structure regulates gene expression is paving the way towards their elucidation.

Besides acetylation, histones undergo many post-translational modifications, such as methylation, phosphorylation, ubiquitination and ribosylation. Exciting recent discoveries have coalesced into the 'histone code' hypothesis. According to this hypothesis, histone modifications, acting alone or in specific combinations, provide binding platforms for chromatin-associated proteins that initiate or block gene transcription [2]. Among the histone modifications implicated in gene silencing, the best characterized to date are histone deacetylation, and methylation of histone H3 at lysine 9 (H3K9me1) [3]. It is increasingly clear that, in various organisms, these modifications work hand-in-hand with DNA methylation to repress transcription [4,5].

This review, which focuses mainly on mammals, provides a concise update on the emerging picture of how DNA methylation, histone deacetylation, and H3K9 methylation contribute jointly to gene silencing. The possibility that CpG methylation might 'converse' with other histone modifications is also discussed.

DNA methylation, histone deacetylation and H3K9 methylation: mutual boosting and feedback loops

The existence of an epigenetic 'conversation' between histones and DNA, involving cytosine methylation, histone deacetylation, and H3K9 methylation, and leading to transcriptional silencing, is now well established. What remains unclear is the precise sequence of events.

On the one hand, there is evidence that DNA methylation influences the histone modification pattern. For instance, early observations showed that components of the DNA methylation machinery (i.e. DNA methyltransferases [DNMTs] and some methyl-CpG-binding domain [MBD] proteins) recruit repressor complexes containing histone deacetylases (HDACs) [6]. Other work using inhibitors of DNA methylation and histone deacetylation showed that CpG methylation is the dominant event that seals transcriptional repression of hypermethylated genes in cancer [7].

On the other hand, some studies suggest that histone modification is a prerequisite for DNA methylation. Observations made in fungi, plants and mammals highlight methylation at lysine 9 of H3 as a kind of 'beacon' for DNA methylation [8,9,10•]. In mammals, DNA methyltransferases interact with Suv39h H3K9 methyltransferases [10•,11], and loss of H3K9 methylation in *Suv39h*-knockout embryonic stem cells decreases Dnmt3b-dependent CpG methylation at major centromeric satellites [10•]. In addition, H3K9 methylation and silencing of the $p16^{ink4a}$ tumor suppressor gene can occur

REVIEW

Form follows function: the genomic organization of cellular differentiation

Steven T. Kosak[1] and Mark Groudine[1,2,3]

[1]Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109, USA; [2]Department of Radiation Oncology, University of Washington School of Medicine, Seattle, Washington 98195, USA

The extent to which the nucleus is functionally organized has broad biological implications. Evidence supports the idea that basic nuclear functions, such as transcription, are structurally integrated within the nucleus. Moreover, recent studies indicate that the linear arrangement of genes within eukaryotic genomes is nonrandom. We suggest that determining the relationship between nuclear organization and the linear arrangement of genes will lead to a greater understanding of how transcriptomes, dedicated to a particular cellular function or fate, are coordinately regulated. Current network theories may provide a useful framework for modeling the inherent complexity the functional organization of the nucleus.

Louis Sullivan, whose early efforts helped pioneer the development of the skyscraper, is considered one of the most important architects of the last century. However, it is his dictum—"form ever follows function"—for which he is perhaps best known. Just as this imperative has influenced generations of architects, the idea that structure reflects function provides a useful perspective for a biologist's view of the cell. In many ways, the structure and function of cellular and subcellular organelles are inseparable; that is, disruptions in organelle function can lead to perturbations in its structure. Upon inhibition of rRNA transcription, for example, the nucleolus becomes disordered and ultimately disappears (Leung and Lamond 2003). This integration of structure and cellular function allows for conservation of resources and facilitates regulation at multiple levels.

Although a completely sequenced genome may represent a genetic blueprint, molecular biologists currently lack a key with which to fully grasp how this sequence is related to the development and subsequent maintenance of a given organism. Following Sullivan's example, a comprehensive understanding of genomic sequence may require considering its arrangement in the nucleus; the form DNA takes in the nucleus reveals not only its higher-order structure, but it may impart information regarding its function. The current paradigm of gene regulation includes the binding of site-specific transcription factors, the recruitment of cofactors and general transcription factors, and the incorporation of multiple modifications to both the DNA and the histones that organize it (Felsenfeld and Groudine 2003). This description of transcription belies its enormous complexity, fueled by an ever-increasing catalog of proteins dedicated in one way or another to its regulation. Additionally, evidence supporting the role of nuclear localization in transcriptional regulation indicates that it is insufficient to know the components of transcription (Francastel et al. 2000). Rather, a thorough understanding of the process requires knowing its functional organization within the nucleus. In this sense, transcription should not be viewed simply as a process that turns on a specific gene, but as a process that governs within the genome an entire network of genes (a transcriptome) that gives rise to a particular cellular function or fate (such as cell division, differentiation, or apoptosis). Therefore, the challenge is to uncover the nuclear organization of gene activity and to determine whether genomes are specifically structured.

The form DNA takes in the nucleus is a result of at least three prevailing components, its organization into chromatin, the linear order of genes and repetitive elements along their respective chromosomes, and the spatial localization of genes and repeats within the nucleus. Current efforts with molecular, cell biological, and genomic approaches are attempting to elucidate the role each of these components of DNA plays in regulating nuclear processes. Clearly, the forms of DNA permissive for gene transcription and gene silencing are of particular importance. This review will survey what is currently known about the localization of genes spatially within the nucleus and linearly in the genome, focusing on how these organizational states may help facilitate the orchestrated gene expression that results in cellular differentiation. Finally, the review will explore how this coordinated expression may be modeled by current network theories.

Spatial organization of gene activity within the nucleus

Cellular differentiation is generally accompanied by coordinated changes in gene expression and alterations in

[*Keywords*: Nuclear structure; genomic organization; transcription; expression neighborhood; genetic networks]
[3]Corresponding author.
E-MAIL markg@fhcrc.org; FAX (206) 667-5894.
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Rules of engagement: co-transcriptional recruitment of pre-mRNA processing factors

David L Bentley

The universal pre-mRNA processing events of 5′ end capping, splicing, and 3′ end formation by cleavage/polyadenylation occur co-transcriptionally. As a result, the substrate for mRNA processing factors is a nascent RNA chain that is being extruded from the RNA polymerase II exit channel at 10–30 bases per second. How do processing factors find their substrate RNAs and complete most mRNA maturation before transcription is finished? Recent studies suggest that this task is facilitated by a combination of protein–RNA and protein–protein interactions within a 'mRNA factory' that comprises the elongating RNA polymerase and associated processing factors. This 'factory' undergoes dynamic changes in composition as it traverses a gene and provides the setting for regulatory interactions that couple processing to transcriptional elongation and termination.

Addresses
Department of Biochemistry and Molecular Genetics, University of Colorado School of Medicine, UCHSC at Fitzsimons, Mail Stop 8101, PO Box 6511, Aurora Colorado 80045, USA

Corresponding author: Bentley, David L (David.Bentley@UCHSC.edu)

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Introduction: how does coupling to transcription enhance pre-mRNA processing?

How mRNA processing is facilitated by coupling to RNA polymerase II (pol II) transcription is a fascinating problem that is the subject of several excellent reviews [1–5]. Transcription-coupled processing differs from uncoupled processing in that the substrate RNA is a growing and progressively folding structure rather than a static full-length pre-mRNA. The importance of coupling is suggested by the fact that processing of full-length synthetic pre-mRNAs in injected oocytes is less efficient than co-transcriptional processing *in vivo* [6]. *In vivo*, introns can be removed and the poly(A) site cleaved by the time polymerase has transcribed only 1 kb beyond the processing sites, probably within 30s [7]. By contrast, *in vitro* processing uncoupled from transcription usually takes >20 min. Optimal processing is achieved by coupling with transcription by RNA pol II and not other polymerases because pol II is uniquely equipped with an unusual domain on its large subunit, called the C-terminal domain (CTD), that provides a landing pad for mRNA processing factors.

Coupling of pol II transcription with processing can influence processing reactions in at least three ways. First, localization: in its simplest form, coupling positions mRNA processing factors at the elongation complex, raising their local concentration in the vicinity of the nascent transcript. Second, kinetic coupling: the rate of transcript elongation can have profound effects on RNA folding and the assembly of RNA–protein complexes and has been shown to affect the choice between alternative processing sites [8,9]. Third, allostery: contacts between mRNA processing factors and the pol II elongation complex can allosterically activate or inhibit mRNA processing factors [10]. Here I will review recent progress in our understanding of how the factors that carry out mRNA capping, splicing and 3′ end formation engage the pol II elongation complex.

The pol II C-terminal domain: a recruitment platform

The CTD is an essential domain in the large subunit of pol II, but is absent from the related subunits of RNA polymerases I and III. This domain comprises tandem heptads whose consensus sequence, $Y_1S_2P_3T_4S_5P_6S_7$, is identical across animals, plants and some protozoa. The *in vivo* functional unit of the CTD appears to be a pair of tandem heptads [11]. A recent proteomic analysis identified over 100 yeast proteins that bind to the phosphorylated CTD [12•]. The CTD is more than a passive landing pad, however. Among its numerous roles, the CTD can allosterically regulate capping enzymes and regulate transcriptional elongation and termination [13].

CTD deletion prevents efficient co-transcriptional capping, splicing and 3′ end formation in metazoans [14,15]. Although it is essential for co-transcriptional processing, the CTD is dispensable for processing uncoupled from transcription in injected *Xenopus* oocytes [6], suggesting that processing at the site of transcription differs from post-transcriptional processing elsewhere in the nucleus. Important clues to how the CTD works has come from *in vitro* systems, in which it can stimulate processing reactions, in some cases even in the absence of ongoing transcription [16,17••].

Chromosome positioning in the interphase nucleus

Luis A. Parada and Tom Misteli

Chromosomes occupy distinct territories in the interphase cell nucleus. These chromosome territories are non-randomly arranged within the nuclear space. We are only just uncovering how chromosome territories are organized, what determines their position and how their spatial organization affects the expression of genes and genomes. Here, we discuss emerging models of non-random nuclear chromosome organization and consider the functional implications of chromosome positioning for gene expression and genome stability.

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Luis A. Parada
Tom Misteli*
National Cancer Inst., NIH, Bethesda, MD 20892, USA.
*e-mail: mistelit@mail.nih.gov

The study of gene expression is rapidly changing. Rather than analysing in detail the molecular mechanisms involved in regulation of single genes, the focus in the genome era is on the global understanding of the genome within the context of the entire nucleus. It is becoming increasingly clear that regulation of gene expression cannot be accounted for only by the information encoded in the regulatory elements contained in the linear DNA sequence. Higher-order chromatin structure and epigenetic regulation via chromatin modifications play crucial roles in gene expression, and accumulating evidence points to nuclear architecture and the spatial organization of the genome as major factors in the regulation of single genes and gene expression programs [1–3]. Higher-order organization of chromatin and chromosomes must therefore be considered important regulatory factors in gene expression. Whereas the rapidly emerging genome-sequence data tell us how genes and regulatory sequences are organized linearly on chromosomes, they reveal little about the spatial organization of these sequences in the cell nucleus or how spatial and temporal genome organization contributes to gene regulation. It is clear that, in order to understand the control mechanisms of gene expression programs *in vivo*, it will be essential to uncover how genomes are spatially and temporally organized.

The natural unit of subdivision of the genome is the chromosome. Although chromosomes can easily be seen in cells during mitosis, when they appear as distinct, highly condensed entities (Fig. 1a), their morphological nature during interphase remained elusive for many years [4,5]. Recent advances in microscopy now allow the routine visualization of chromosomes in the interphase nucleus (Fig. 1b,c). These techniques, in conjunction with the availability of complete genome data, permit for the first time the localization of any part of the genome in space and time and are providing first insights into the rules that govern chromosome organization during interphase. Here, we discuss emerging models of non-random chromosome positioning and their functional implications.

Chromosome territories

The genetic material that makes up a single chromosome is not distributed in a disorderly fashion throughout the interphase nucleus. Instead, each chromosome occupies a finite, mutually exclusive fraction of the nuclear volume and represents a structural unit referred to as a chromosome territory (Fig. 1b,c). The territorial organization of chromosomes in interphase cells was originally proposed by Rabl and Boveri more than a century ago, and was confirmed in the 1980s by Cremer and colleagues using elegant ultraviolet-laser microirradiation experiments [6]. The term chromosome territory was coined to reflect the distinct physical nature of chromosomes in the interphase nucleus.

Chromosome territories can now be visualized directly by *in situ* hybridization using fluorescently labelled probes addressed to single chromosomes (Fig. 1b,c). Fluorescent *in situ* hybridization (FISH) studies have unequivocally established the existence of chromosomes as individual territories in higher organisms [7,8]. High-resolution light microscopy and electron microscopy clearly demonstrate that chromosome territories are distinct entities that show no significant intermingling (Fig. 1c) [9–11]. The size of a chromosome territory is roughly determined by its DNA content but is also affected by other factors such as its overall transcriptional status [12,13].

Although the internal organization of chromosome territories is still unclear, some aspects of their ultrastructural organization have been elucidated. Metaphase chromosomes display a chromosome-specific series of alternating light- and dark-staining bands (R- and G-bands), corresponding to early- and late-replicating regions of the genome. During interphase, these regions are maintained and occupy distinct domains within a chromosome territory [10,14,15]. The replication domains are ~1 Mb in size and constitute a distinct level of chromosome territory organization, because they are maintained during consecutive cell cycles [16,17]. The chromatin fibre within chromosome territories has been suggested either to exist in the form of loops of 30–150 kb, which in turn form rosettes to give the 1 Mb

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Recent highlights of RNA-polymerase-II-mediated transcription

Robert J Sims III, Subhrangsu S Mandal and Danny Reinberg[1]

Considerable advances into the basis of RNA-polymerase-II-mediated transcriptional regulation have recently emerged. Biochemical, genetic and structural studies have contributed to novel insights into transcription, as well as the functional significance of covalent histone modifications. New details regarding transcription elongation through chromatin have further defined the mechanism behind this action, and identified how chromatin structure may be maintained after RNAP II traverses a nucleosome. ATP-dependent chromatin remodeling complexes, along with histone chaperone complexes, were recently discovered to facilitate histone exchange. In addition, it has become increasingly clear that transcription by RNA polymerase II extends beyond RNA synthesis, towards a more active role in mRNA maturation, surveillance and export to the cytoplasm.

Addresses
Howard Hughes Medical Institute, Division of Nucleic Acids Enzymology, Department of Biochemistry, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, NJ 08854, USA
[1]e-mail: reinbedf@UMDNJ.EDU

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Abbreviations

DSIF	DRB sensitivity inducing factor
FACT	facilitates chromatin transcription
HIRA	histone regulatory homolog A
HMTase	histone methyltransferase
NELF	negative elongation factor
P-TEFb	positive transcription elongation factor
RNAP II	RNA polymerase II
RSC	remodels the structure of chromatin
SAGA	Spt-Ada-Gcn5-Acetyltransferase
SCP	small CTD phosphatases
SPT	Suppressor of Ty insertion
SWR	Swi2/Snf2 related
TF	transcription factor

Introduction

Over the past few years, the current model of gene regulation has acquired a new degree of sophistication, as advances in our understanding of transcriptional regulation by RNA polymerase II (RNAP II) have developed at a staggering pace. Diverse studies of the transcriptional machinery have provided a detailed view of how the general transcription factors work together to transcribe protein-coding genes efficiently. Importantly, recent advances in the field of gene regulation have more clearly demonstrated that transcription is coupled to mRNA processing, RNA surveillance and export, among other cellular processes. The consequences of DNA accessibility represent a major challenge in our attempts to understand how genes are transcriptionally regulated. Great strides have been made regarding the mechanisms behind RNAP II transcription in a chromatin environment. Significant advancements in our understanding of the language of covalent histone modifications have deepened our knowledge of the information stored within nucleosomes, the building blocks of chromatin. Here we discuss recent advances in RNAP II transcription, including structural aspects, mRNA processing and export, elongation through chromatin, histone modifications, and the recent discoveries of histone exchangers.

The transcriptional machinery

The high-resolution crystal structure of RNAP II has provided detailed insight at the atomic level into how a catalytically active RNAP II is structured and has revealed important aspects of its function [1,2]. Most importantly, significant homology exists between eukaryotic and bacterial RNA polymerases in their overall structure [3]. The relative positions of the conserved subunits of RNAP II, including Rpb1(β′), Rpb2(β), Rpb3(αI), Rpb11(αII) and Rpb6(ω), are consistent in yeast and bacteria, suggesting the existence of an evolutionarily conserved mechanism of RNA synthesis during transcription. Notably, the nascent mRNA passes through a positively charged exit channel, and once the RNA is approximately 18 nucleotides long it becomes accessible to the RNA processing machinery. These structural observations are consistent with the coupling of transcript capping to early transcription events. Furthermore, several additional structural, biochemical and genetic studies conducted over the past few years have yielded important details of RNAP II function [2,4].

Transcription of class II genes requires a coordinated assembly of RNAP II and five factors, TFIID, TFIIB, TFIIF, TFIIE and TFIIH, the so-called general transcription factors [5,6]. Transcription initiation begins with the formation of the first phosphodiester bond and phosphorylation of serine 5 (Ser5) on the C-terminal domain (CTD) of the largest subunit of RNAP II by TFIIH. The CTD of RNAP II, composed of a highly conserved, tandemly repeated heptapeptide motif (YSPTSPS), undergoes extensive phosphorylation and dephosphorylation during the transcription cycle. The oscillation of

CONTROLLING NUCLEAR RECEPTORS: THE CIRCULAR LOGIC OF COFACTOR CYCLES

Valentina Perissi and Michael G. Rosenfeld

Abstract | Nuclear receptors regulate many biologically important processes in development and homeostasis by their bimodal function as repressors and activators of gene transcription. A finely tuned modulation of the transcriptional activities of nuclear receptors is crucial for determining highly specific and diversified programmes of gene expression. Recent studies have provided insights into the molecular mechanisms that are required to switch between repression and activation functions, the combinatorial roles of the multiple cofactor complexes that are required for mediating transcriptional regulation, and the central question of how several different signalling pathways can be integrated at the nuclear level to achieve specific profiles of gene expression.

ORPHAN RECEPTOR
A subclass of nuclear receptors that were originally identified as orphans because the ligand was unknown.

Howard Hughes Medical Institute, Department of Medicine, University of California San Diego, 9500 Gilman Drive, La Jolla, California 92093-064, USA.
e-mails: vperissi@ucsd.edu; mrosenfeld@ucsd.edu
doi:10.1038/nrm1680

Precise spatial and temporal patterns of gene expression are crucial for the normal development of all organisms. Orchestrating these patterns requires the coordination of numerous regulatory events and mechanisms that mediate both the repression and activation of specific target genes at specific times and places during development. DNA-binding transcription factors, non-DNA-binding coregulators and general components of the basal RNA polymerase machinery are essential to regulate transcription and achieve the correct patterns of gene expression. Although there are many variations in their functions and in the highly specific strategies of regulation, several general themes and common rules have emerged.

Nuclear receptors provide an interesting model to study the specific, as well as the more general, mechanisms of transcriptional regulation; they are highly regulated DNA-binding transcription factors that are directly modulated by ligand binding and can both activate and repress gene expression. Here, we review several recent advances in our understanding of nuclear-receptor-mediated transcriptional regulation by focusing on the dedicated mechanisms that regulate the switch from gene repression to gene activation and that further modulate transcriptional activity. The complexity of regulatory proteins and the many ways of modulating their recruitment and their activity tightly regulates this apparently simple, but fascinating, recruitment event.

The nuclear receptor superfamily: an overview

The mammalian nuclear receptor superfamily comprises more than 45 transcription factors, many of which regulate gene expression in a ligand-dependent manner. Members of the nuclear receptor superfamily include: receptors for steroid hormones, such as the oestrogen receptor (ER), the androgen receptor (AR) and the glucocorticoid receptor (GR); receptors for non-steroidal ligands, such as the thyroid hormone receptor (TR) and the retinoic acid receptor (RAR); as well as receptors that bind diverse products of lipid metabolism such as fatty acids and prostaglandins (peroxisome proliferator activated receptors (PPARs) and liver X receptors (LXRs))[1,2]. The nuclear receptor superfamily also includes many so-called ORPHAN RECEPTORS for which regulatory ligands are still unknown or for which candidates have only recently been identified by screening strategies that

REPLICATION AND TRANSCRIPTION: SHAPING THE LANDSCAPE OF THE GENOME

Lyubomira Chakalova, Emmanuel Debrand, Jennifer A. Mitchell, Cameron S. Osborne and Peter Fraser

Abstract | As the relationship between nuclear structure and function begins to unfold, a picture is emerging of a dynamic landscape that is centred on the two main processes that execute the regulated use and propagation of the genome. Rather than being subservient enzymatic activities, the replication and transcriptional machineries provide potent forces that organize the genome in three-dimensional nuclear space. Their activities provide opportunities for epigenetic changes that are required for differentiation and development. In addition, they impose physical constraints on the genome that might help to shape its evolution.

Laboratory of Chromatin and Gene Expression, The Babraham Institute, Babraham Research Campus, Cambridge CB2 4AT, United Kingdom. Correspondence to P.F. e-mail: peter.fraser@bbsrc.ac.uk
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Based on studies of a small number of individual genes, it has been known for decades that there is a correlation between transcription and DNA replication in early S phase. Active genes tend to replicate early in S phase whereas inactive genes replicate late[1,2]. Replication imposes the need to re-establish epigenetic information on both daughter fibres of newly replicated chromatin so that cells can continue their specific gene-expression programmes for the remainder of the current cell cycle, and inherit or remember those patterns in the next cell cycle. This has led to the speculation that chromatin assembly at replication forks could specify gene-expression states (see REF. 3 and references therein). In particular, it was proposed that early replication could specify permissive chromatin states that allow gene expression, whereas late replication could specify heterochromatin assembly at silent regions. Recent large-scale studies have confirmed a strong correlation between early DNA replication and gene expression, fostering the link between transcription and replication timing[4,5]. However, these studies also uncovered many expressed genes that replicate late and many inactive genes that replicate early. So what is the link between transcription and replication timing? An appreciation of the structure of the genome and its organization in the nucleus relative to the transcription and replication machineries, coupled with revealing findings on the increased complexity of the transcriptome, indicates a model that could explain the interplay between these processes.

Transcription has long been thought to be the primary regulatory step in controlling most gene-expression programmes in differentiation and development. Much attention over the years has focused on the control of mRNA expression, which is the product of protein-coding genes. However, recent work shows that mRNA, and in particular polyadenylated mRNA, is the least complex RNA population in cells[6]. Analysis of the human transcriptome has shown that transcription occurs over much wider areas of the genome than can be accounted for by protein-coding genes[6,7]. This realization, coupled with an appreciation that transcription is highly compartmentalized within the nucleus, indicates that the transcriptional machinery has a central role in the organization of the genome.

In this review we focus on the spatial and temporal control of DNA replication and transcription, pointing out how these processes both reflect and shape the nuclear landscape of the genome. We relate the

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Biological Control through Regulated Transcriptional Coactivators

Review

Bruce M. Spiegelman[1,*] and Reinhart Heinrich[2]
[1]Dana-Farber Cancer Institute and Department of Cell Biology
Harvard Medical School
Boston, Massachusetts 02115
[2]Institute of Biology
Section of Theoretical Biophysics
Humboldt University
10115 Berlin
Germany

Gene activation in higher eukaryotes requires the concerted action of transcription factors and coactivator proteins. Coactivators exist in multiprotein complexes that dock on transcription factors and modify chromatin, allowing effective transcription to take place. While biological control focused at the level of the transcription factor is very common, it is now quite clear that a substantial component of gene control is directed at the expression of coactivators, involving pathways as diverse as B-cell development, smooth muscle differentiation, and hepatic gluconeogenesis. Quantitative control of coactivators allows the functional integration of multiple transcription factors and facilitates the formation of distinct biological programs. This coordination and acceleration of different steps in linked pathways has important kinetic considerations, enabling outputs of particular pathways to be increased far more than would otherwise be possible. These kinetic aspects suggest opportunities and concerns as coactivators become targets of therapeutic intervention.

Introduction

Gene activation is a multistep process involving a very large number of proteins functioning in discrete complexes. Transcription factors bind to DNA in a sequence-specific manner and essentially mark a gene for activation or repression through the recruitment of coactivator or corepressor proteins. Coactivators function in a variety of ways and often contain the enzymatic activities necessary for an alteration in chromatin structure from a quiescent state to one allowing active gene transcription. Broadly speaking, coactivators can be thought of in three classes. One class of proteins modifies histones in ways that allow greater access of other proteins to the DNA. Examples of these are p300 and CBP, powerful histone acetyltransferases (HATs) that interact with a wide variety of transcription factors and other proteins (Hermanson et al., 2002). These proteins support transcription in vitro from chromatinized templates. A second class of coactivators are members of the TRAP/DRIP/Mediator/ARC complex, proteins that bind to transcription factors, recruit RNA polymerase II and interact with the general transcription apparatus. The Mediator complex supports transcription in vitro from DNA templates but does not support efficient transcription from chromatinized templates (reviewed in Malik and Roeder, 2000). Lastly, protein complexes of the yeast SWI/SNF family (or their mammalian homologs BRG1 or BRM) contain ATP-dependent DNA unwinding activities, necessary for efficient gene transcription in vivo. These groups of proteins will not support transcription from naked DNA, but augment transcription from chromatinized templates in vitro (Lemon et al., 2001). More biochemical activities of coactivator proteins are very likely to be discovered.

Corepressors have the opposite effect on chromatin structure, making it inaccessible to the binding of transcription factors or resistant to their actions. These proteins (such as NcoR) are often associated with histone deacetylase (HDAC) activity, though other mechanisms for gene silencing clearly exist (Hermanson et al., 2002).

Although coactivators are defined as proteins that increase transcriptional activity without binding to DNA, it is useful to think of those that bind directly to transcription factors and contain relevant enzymatic activities as primary coactivators (Figure 1). Those that dock on transcription factors and serve as scaffolds for the recruitment of other proteins containing these enzymatic activities can be considered secondary coactivators. As shown in Figure 1 and discussed below, this distinction rapidly blurs as proteins that can function as primary coactivators on some transcription factors can also be used as enzymatic tools assembled by secondary coactivators in other contexts.

The regulation of gene activity at the transcriptional level has generally been thought to occur via changes in amounts or activities of transcription factors. In this view, transcription factors may themselves be transcriptionally induced or repressed, and activated or deactivated by proteolysis, covalent modification, or ligand binding. The control of nuclear NFκB through pathways of signal transduction in inflammation and the induction of the myogenic b-HLH proteins, such as MyoD, during muscle differentiation are classic examples of biological control through modulation of transcription factor quantity.

However, recent data has shown that important physiological control of gene regulatory systems is not solely the province of transcription factors. Coactivator proteins may participate in gene regulation, not merely by being necessary gears in the transcriptional machinery, but by being the *primary* targets of developmental or physiological signals. In this review, we will discuss data illustrating complex biological processes controlled at the coactivator level. While posttranslation modification of coactivator proteins occurs and represents an emerging area of discovery, we will concentrate on several examples of modulation of coactivator expression in various biological settings. We have chosen to discuss in detail those examples where the coactivation function of a given protein or set of proteins is very well established, and where the regulation in physiological settings is a key part of the significance of that coactivator. This

*Correspondence: bruce_spiegelman@dfci.harvard.edu

REVIEW

RNA meets chromatin

Emily Bernstein and C. David Allis[1]

Laboratory of Chromatin Biology, The Rockefeller University, New York, New York 10021, USA

In the universe of science, two worlds have recently collided—those of RNA and chromatin. The intersection of these two fields has been impending, but evidence for such a meaningful collision has only recently become apparent. In this review, we discuss the implications for noncoding RNAs and the formation of specialized chromatin domains in various epigenetic processes as diverse as dosage compensation, RNA interference-mediated heterochromatin assembly and gene silencing, and programmed DNA elimination. While mechanistic details as to how the RNA and chromatin worlds connect remain unclear, intriguing parallels exist in the overall design and machinery used in model organisms from all eukaryotic kingdoms. The role of potential RNA-binding chromatin-associated proteins will be discussed as one possible link between RNA and chromatin.

Chromatin, the intimate association of histone proteins and DNA into repeating nucleosomal units, is the physiologically relevant structure of our genome. An increasing body of evidence suggests that variation can be introduced into the chromatin polymer by an elaborate set of mechanisms that fail to alter the DNA template itself. The inheritance of chromatin states such as "active" (euchromatic) or "silent" (heterochromatic) domains forms the foundation of epigenetics. Until recently, understanding how, if at all, noncoding RNAs fit into the chromatin world, by influencing either euchromatin or heterochromatin, remained a puzzle that most biologists had simply not considered. Although the underlying mechanisms linking RNA and chromatin remain unclear, understanding how these epigenetic states are established and maintained during the life of a cell or development of an organism is imperative.

We favor the general view that a complete appreciation of epigenetic regulation is likely to require a careful examination of both RNA and chromatin fields. One goal of this review is to expose potential links between these two research areas, with a focus on transcriptional gene silencing in a wide range of experimental models. We conclude with a speculative model for how a group of heterochromatin-associated proteins may participate in linking RNA and chromatin.

[*Keywords*: RNAi; dosage compensation; heterochromatin; transcriptional gene silencing]
[1]Corresponding author.
E-MAIL alliscd@mail.rockefeller.edu; FAX (212) 327-7849.
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Chromatin dynamics

The nucleosome is the fundamental repeating subunit of chromatin, consisting of an octamer of histone proteins (two copies of each of the four core histones H2A, H2B, H3, and H4) around which a defined segment (147 base pairs) of DNA is wound (Luger et al. 1997). Relatively unstructured histone "tail" domains are exposed on the nucleosomal surface and are rich in post-translational modifications such as serine and threonine phosphorylation, lysine acetylation, lysine and arginine methylation, lysine ubiquitination and sumoylation, and ADP-ribosylation (Strahl and Allis 2000; Zhang and Reinberg 2001). Emerging evidence suggests that covalent marks also exist in the histone-fold domains that correlate with the lateral surface of nucleosomes, near where critical histone:DNA interactions occur. Such modifications may mediate other aspects of chromatin structure (Cosgrove et al. 2004). Regardless of position or function, these covalent marks can be placed and removed by a variety of site-specific enzymes such as histone acetyltransferases (HATs) and histone deacetylases (HDACs), histone methyltransferases (HMTs), and the newly discovered "demethylating" activities (Cuthbert et al. 2004; Shi et al. 2004; Wang et al. 2004), ubiquitin ligases and deubiquitinases, and so on. Additional complexity and biological readout options are provided by the fact that certain modifications, such as methylation, can exist in mono-, di- and trimethylated forms, each of which is placed by a specific HMT. Finally, the notion that patterns of histone modifications can either coexist, on the same tail (*cis*) or on distinct tails (*trans*) that mediate potential "cross-talk" or "switching" between distinct marks and their binding partners (see below), has been formally proposed (Jenuwein and Allis 2001; Fischle et al. 2003a) and is currently under active investigation.

The sheer complexity of covalent histone modifications is multiplied by the existence of histone variants in many organisms, that give the cell added opportunities to change the overall composition of the nucleosome and its covalent modification potential (for review, see Kamakaka and Biggins 2005). In either the case of conventional or variant histones, the fundamental question of

the inability of these cells to recover from the hydroxyurea treatment. We did not detect abnormal DNA structures at early replicating origins in *rad53* cells grown under normal conditions (Fig. 3c and data not shown), and therefore we must assume that hydroxyurea treatment greatly amplifies the presence of these abnormal intermediates. Although our approach may not be sensitive enough to detect a small amount of these structures, it is also possible that replication forks in the 305-rf in *rad53* cells grown under normal conditions will never collapse, but rather that this event is restricted to natural pause sites in the genome[25,26] or sites where the forks encounter a damaged template. From this perspective, we propose that the checkpoint response directly modulates the stability of replicating chromosomes, thus contributing to the prevention of genomic rearrangements, which are the most prominent hallmarks of cancer susceptibility in multicellular organisms. □

Methods

We used the following strains: W303-1A[27] (*MAT**a** ade2-1*, *trp1-1*, *leu2-3*, *112 hys3-11*, *15 ura3*, *can1-100*) and its isogenic derivatives CY2034 (*rad53-K227A-KanMX4*), CY387 (*pri1-M4*), CY2059 (*pri2-1*) and CY2061 (*cdc17-1*)[6,28]. Strains CY2572 (vector) and CY2573 (*GAL1rad53*) were constructed by integrating in the W303-1A strain, respectively, the YIplac128 (*LEU2*) vector plasmid or its pCH12 derivative[6], containing the *Eco*RI fragment carrying the *rad53-D339A* mutant allele under the control of the *GAL1* promoter. Strains CY3278 (*mec1-td*) and CY3281 (*mec1-td*, *rad53-K227A*) are isogenic to W303 and were constructed by replacing the wild-type copy of *MEC1* with the *mec1-*^{ts}*degron* allele as already described[29].

Yeast protein extracts prepared by the TCA extraction method[6] were resolved by 10% SDS–PAGE, and the phosphorylation state of the Rad53 polypeptide was analysed by western blotting using anti-Rad53 antibodies (provided by C. Santocanale and J. Diffley). DNA samples to be analysed with the neutral–neutral two-dimensional electrophoresis technique were prepared and analysed essentially as described[17]: first-dimension gels were 0.35% agarose and second-dimension gels were 0.9% agarose. Replication intermediates were quantified as already described[26], by calculating the percentage of the specific replication-intermediate signals relatively to the monomer spot. FACS analysis was performed as described[6].

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Correspondence and requests for materials should be addressed to M.F. (e-mail: foiani@ifom-firc.it).

Rb targets histone H3 methylation and HP1 to promoters

Soren J. Nielsen*†, Robert Schneider*†, Uta-Maria Bauer*, Andrew J. Bannister*, Ashby Morrison‡, Donal O'Carroll§, Ron Firestein‖, Michael Cleary‖, Thomas Jenuwein§, Rafael E. Herrera‡ & Tony Kouzarides*

** Wellcome/CRC Institute and Department of Pathology, Tennis Court Road, Cambridge CB2 1QR, UK*
‡ Baylor College of Medicine, Department of Molecular and Cellular Biology, The Breast Center, 1 Baylor Plaza, Houston, Texas 77030, USA
§ Research Institute of Molecular Pathology (IMP), The Vienna Biocenter, Dr. Bohrgasse 7, A-1030 Vienna, Austria
‖ Department of Pathology, Stanford University Medical Center, Stanford, California 94305, USA
† These authors have contributed equally to this work.

In eukaryotic cells the histone methylase SUV39H1 and the methyl-lysine binding protein HP1 functionally interact to repress transcription at heterochromatic sites[1]. Lysine 9 of histone H3 is methylated by SUV39H1 (ref. 2), creating a binding site for the chromo domain of HP1 (refs 3, 4). Here we show that SUV39H1 and HP1 are both involved in the repressive functions of the retinoblastoma (Rb) protein. Rb associates with SUV39H1 and HP1 *in vivo* by means of its pocket domain. SUV39H1 cooperates with Rb to repress the cyclin E promoter, and in fibroblasts that are disrupted for SUV39, the activity of the cyclin E and cyclin A2 genes are specifically elevated. Chromatin immunoprecipitations show that Rb is necessary to direct methylation of histone H3, and is necessary for binding of HP1 to the cyclin E promoter. These results indicate that the SUV39H1–HP1 complex is not only involved in heterochromatic silencing but also has a role in repression of euchromatic genes by Rb and perhaps other co-repressor proteins.

The Rb protein functions as a repressor, at least partly, through the recruitment of histone deacetylase activity[5–7]. We considered whether histone methylation might also be involved in Rb-mediated

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Estrogen Receptor-α Directs Ordered, Cyclical, and Combinatorial Recruitment of Cofactors on a Natural Target Promoter

Raphaël Métivier,[1,2,4,*] Graziella Penot,[1,2] Michael R. Hübner,[1] George Reid,[1] Heike Brand,[1] Martin Koš,[1,3] and Frank Gannon[1,*]
[1]European Molecular Biology Laboratory (EMBL)
Meyerhofstrasse 1
D-69117 Heidelberg
Germany

Summary

Transcriptional activation of a gene involves an orchestrated recruitment of components of the basal transcription machinery and intermediate factors, concomitant with an alteration in local chromatin structure generated by posttranslational modifications of histone tails and nucleosome remodeling. We provide here a comprehensive picture of events resulting in transcriptional activation of a gene, through evaluating the estrogen receptor-α (NR3A1) target pS2 gene promoter in MCF-7 cells. This description integrates chromatin remodeling with a kinetic evaluation of cyclical networks of association of 46 transcription factors with the promoter, as determined by chromatin immunoprecipitation assays. We define the concept of a "transcriptional clock" that directs and achieves the sequential and combinatorial assembly of a transcriptionally productive complex on a promoter. Furthermore, the unanticipated findings of key roles for histone deacetylases and nucleosome-remodeling complexes in limiting transcription implies that transcriptional activation is a cyclical process that requires both activating and repressive epigenetic processes.

Introduction

Estrogens have pivotal functions in both female and male physiology. In particular, 17β-estradiol (E_2) has a central role in the proliferation and differentiation of responsive cells, through changing the expression profile of target genes within responsive tissues (Feigelson and Henderson, 1996; Nilsson et al., 2001). The effects of E_2 are mediated through binding to transcription factors belonging to the nuclear receptor (NR) superfamily, the estrogen receptors (ERα and ERβ). ERs activate transcription through associating with estrogen-responsive elements (ERE) located within the promoter regions of target genes (Robinson-Rechavi et al., 2003). However, transcription is achieved within a restrictive environment, as the packaging of promoter DNA and histones into nucleosomes precludes gene expression (Wolffe, 1992; Dillon and Festenstein, 2002). The specific and ordered recruitment and assembly of several multisubunit protein complexes on promoters provides chromatin with the plasticity required for transcription initiation (Cosma, 2002; Narlikar et al., 2002). Accordingly, ERα undergoes major structural rearrangements on association of ligand to expose binding surfaces that recruit transcription cofactors (Brzozowski et al., 1997). In the last 10 years, a plethora of cofactors involved in ER-mediated transactivation have been identified (Klinge, 2000; McKenna and O'Malley, 2002; Belandia and Parker, 2003). Among these proteins are the SWI/SNF complexes (Belandia et al., 2002) that alter the spatial organization of nucleosomes in an ATP-dependent manner (Kassabov et al., 2003). Other enzymes recruited by ER modify the lysine or arginine residues of histone tails (Lee et al., 2001), processes necessary for chromatin remodeling (Berger, 2002; Marmorstein, 2001). Histones acetyl-transferases (HAT) conscripted by ER include members of the p160 subfamily such as SRC1 (Onate et al., 1995) or the integrator complexes p300/CBP and p/CAF (Lee et al., 2001). Histone methyl-transferase (HMT) activity, provided by proteins such as CARM1 or PRMT1, also poise a promoter for transcription through methylation of arginine groups within histones (Marmorstein, 2001). Consequently, the nucleosomal architecture of estrogen-responsive promoters is modified following ER binding and subsequent recruitment of protein complexes (Nye et al., 2002).

Induction of transcription requires the formation of the preinitiation complex (PIC), which comprises the six $TF_{II}A$ to F complexes and RNA polymerase II (Pol II; Berk, 1999) on the promoter. Following many years of investigation, a model emerged (Orphanides, et al., 1996; Ogbourne and Antalis, 1998; Emerson, 2002) postulating that recruitment of TBP, a subunit of $TF_{II}D$ that binds the TATA box, first becomes stabilized by $TF_{II}A$. $TF_{II}B$ next joins the complex, assisting in the selection of the initiation site, followed by RNA Pol II once the recruitment of $TF_{II}B$ has structurally remodeled the PIC (process of isomerization). Subsequent initiation of transcription involves recruitment and structural remodeling of the TRAP/mediator complex, which stimulates phosphorylation of the largest subunit of Pol II (Rbp1, or CTD) by $TF_{II}H$ (Malik and Roeder, 2000; Davis et al., 2002; Woychik and Hampsey, 2002). This event provokes exchange of mediator by elongator complexes (Otero et al., 1999), thereby allowing transcription to initiate. ER is known to contact $TF_{II}B$, $TF_{II}E$, and $TF_{II}F$ and subunits of $TF_{II}D$ (TAFs and the TBP; Sabbah et al., 1998; Wu et al., 1999), providing mechanistic evidence for a direct role for ER in transcriptional activation.

Apart from the sequential recruitment of HATs and TRAP/mediator complexes on E_2-responsive genes (Shang et al., 2000, Burakov et al., 2002), no experimental data yet describe the chronological sequence of events that occur during the initiation of transcription. Further, responses to E_2 have to be constrained in their duration, enabling cells to sense E_2, respond, and then

*Correspondence: raphael.metivier@embl-heidelberg.de (R.M.), frank.gannon@embo.org (F.G.)
[2]Present address: Equipe EMR, UMR CNRS 6026 (ICM), Université de Rennes, I. Batiment 13, 35042 Cedex, Rennes, France.
[3]Present address: Wellcome Trust Centre for Cell Biology, University of Edinburgh. Mayfield Road, Edinburgh EH9 3JR, United Kingdom.
[4]As of January 15, 2004, R.M. can be contacted at a new email address: raphael.metivier@univ-rennes1.fr

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Correspondence and requests for materials should be addressed to M.P.S. (e-mail: ms2001@columbia.edu).

TFIIH is negatively regulated by cdk8-containing mediator complexes

Sasha Akoulitchev, Sergei Chuikov & Danny Reinberg*

Howard Hughes Medical Institute, Division of Nucleic Acids Enzymology, Department of Biochemistry, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854, USA

The mammalian cyclin-dependent kinase 8 (cdk8)[1] gene has been linked with a subset of acute lymphoblastic leukaemias[2], and its corresponding protein has been functionally implicated in regulation of transcription[3,4]. Mammalian cdk8 and cyclin C, and their respective yeast homologues, Srb10 and Srb11, are components of the RNA polymerase II holoenzyme complex[5,6] where they function as a protein kinase that phosphorylates the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II (ref. 7). The yeast *SRB10* and *SRB11* genes have been implicated in the negative regulation of transcription[8]. The cdk8/cyclin C protein complex is also found in a number of mammalian Mediator-like protein complexes[3,5,9–12], which repress activated transcription independently of the CTD *in vitro*[9,10]. Here we show that cdk8/cyclin C can regulate transcription by targeting the cdk7/cyclin H subunits of the general transcription initiation factor IIH (TFIIH). cdk8 phosphorylates mammalian cyclin H in the vicinity of its functionally unique amino-terminal and carboxy-terminal α-helical domains[13]. This phosphorylation represses both the ability of TFIIH to activate transcription and its CTD kinase activity. In addition, mimicking cdk8 phosphorylation of cyclin H *in vivo* has a dominant-negative effect on cell growth. Our results link the Mediator complex and the basal transcription machinery by a regulatory pathway involving two cyclin-dependent kinases. This pathway appears to be unique to higher organisms.

The stimulation of gene-specific transcription by transcriptional activator proteins requires coactivators—molecules that mediate communication between the general transcription machinery and the activators. Some transcription coactivators are activator- or gene-specific, whereas others are required for more global gene transcription (general coactivators). General coactivators include PC4, TFIIA and components of the TFIID and RNA polymerase II (RNAPII) holoenzyme complexes. Coactivators in the RNAPII holoenzyme appear to be modular in nature, forming distinct subcomplexes that are capable of positively or negatively regulating transcription[12]. Distinct coactivator complexes have been isolated from mammalian cells. One such complex is NAT[9], which appears to be functionally similar to the mammalian SMCC–TRAP complex[10] and which contains subsets of polypeptides present in other mammalian coactivator complexes such as Mediator[11], DRIP[14], CRSP[15] and ARC[16]. A unique feature of NAT, Mediator and SMCC–TRAP complexes is the presence of cdk8 and cyclin C, which are the human homologues of yeast Srb10 and Srb11, respectively. The Srb10/11 complex and the NAT complex downregulate transcription by phosphorylating the CTD of RNAPII before its association with transcription initiation complexes[7,9]; however, NAT also downregulates transcription independently of the CTD of RNAPII[9]. Notably, other cdk8-containing complexes, such as SMCC–TRAP and Mediator, can function as coactivators of transcription when the reconstituted system is supplemented with a crude protein fraction[11] or when the system is reconstituted with PC4 in the absence (or with limiting amounts) of TFIIH[10].

To investigate further the mechanism by which cdk8 negatively regulates transcription, we isolated the NAT and SMCC–TRAP complexes[9,10], and analysed their effects on transcription as a function of TFIIH (Fig. 1a). In the absence of TFIIH, both complexes enhanced VP16-mediated activation of transcription. However, both complexes mediated the repression of activated transcription in the TFIIH-dependent assay. Notably, a NAT complex containing a kinase-deficient mutant of cdk8 (D173A) did not support repression (Fig. 1b–d). Treatment of immunoadsorbed, highly purified TFIIH with recombinant cdk8/cyclin C under kinase conditions impaired transcription in a phosphatase-reversible manner (Fig. 1e), which indicates that the cdk8 kinase can downregulate transcription by phosphorylating TFIIH.

The transcription system described above requires PC4, which is downregulated by phosphorylation[17], and it has been suggested that cdk8 targets PC4 (ref. 10). To deduce the *in vivo* target of cdk8, we reconstituted activated transcription in the absence of PC4 using a coactivator complex devoid of cdk8; cdk8 was added exogenously to the assays. We purified and analysed Mediator-like complexes that were devoid of cdk8 but contained polypeptides found in other coactivator complexes (see Supplementary Information). The Mediator-like complex displayed properties similar to those

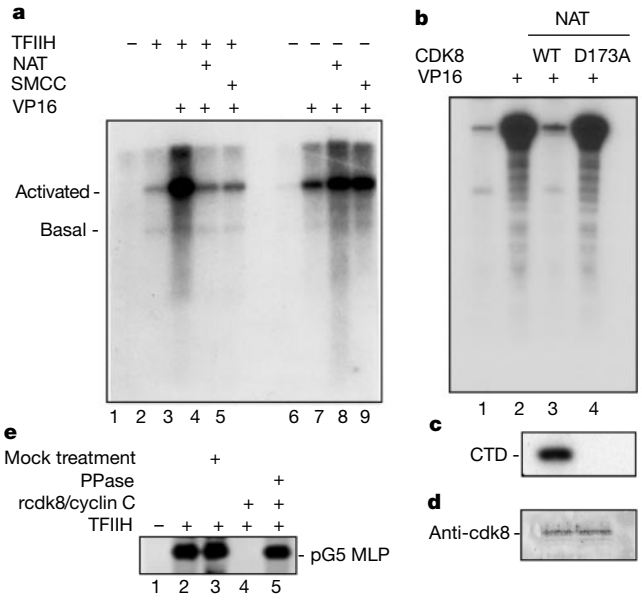

Figure 1 TFIIH mediates cdk8-dependent repression by NAT. **a**, TFIIH-dependent activities of NAT and SMCC complexes. NAT and SMCC–TRAP complexes were purified as described[9,10], and their activities analysed using a reconstituted VP16-dependent transcription activation assay in the presence of PC4. Linear (lanes 1–5) or supercoiled (lanes 6–9) DNA templates were used in the assays, and transcription activity was measured in the presence and absence of TFIIH, respectively. The supercoiled template was used to render the assay independent of TFIIH[29]. The positions of the activated and basal transcription products are indicated. **b**–**d**, Repression by NAT is cdk8 dependent. NAT was immunopurified from 293T cells expressing wild-type cdk8 (WT) or the kinase-deficient mutant[3] of cdk8 (D173A). The preparation was assayed by TFIIH-dependent activated transcription (**b**), CTD kinase assay (**c**) and western blot (**d**). **e**, Cdk8 phosphorylates and inactivates TFIIH. PPase, alkaline phosphatase.

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Ras Induces Mediator Complex Exchange on C/EBPβ

Xianming Mo, Elisabeth Kowenz-Leutz, Hong Xu, and Achim Leutz*
Max-Delbrück-Center for Molecular Medicine
Robert-Roessle-Str. 10
13092 Berlin
Germany

Summary

C/EBPβ is an intrinsically repressed transcription factor that regulates genes involved in differentiation, proliferation, tumorigenesis, and apoptosis. C/EBPβ acts as a repressor that is turned into an activator by the Ras oncoprotein through phosphorylation of a MAPK site. C/EBPβ activation is accompanied by a conformational change. Active and repressive C/EBPβ interacts with multisubunit Mediator complexes through the CRSP130/Sur2 subunit. The CRSP130/Sur2 subunit is common to two distinct types of Mediator complexes, characterized by CRSP70 and CDK8 proteins as transcriptionally active and inactive Mediator, respectively. Knockdown of CRSP130/Sur2 prevents Mediator binding and transactivation through C/EBPβ. Oncogenic Ras signaling or activating mutations in C/EBPβ selects the transcriptionally active Mediator complex that also associates with RNA polymerase II. These results show that a Ras-induced structural alteration of C/EBPβ determines differential gene activation through selective interaction with distinct Mediator complexes.

Introduction

Eukaryotic gene expression is regulated by transcription factors that recognize *cis*-regulatory elements on DNA and that interact with various cofactors that alter chromatin structures and/or mediate contact to the basic transcription machinery (Emerson, 2002; Lemon and Tjian, 2000; Peterson and Workman, 2000; Woychik and Hampsey, 2002). Differential gene expression entails sequential interactions between transcription factors and cofactors in response to signals that emanate from the cell surface. The coordination of signal-dependent and temporal interactions between transcription factors and multiprotein complexes is a central problem in understanding how communication between cells and their environment is transformed into gene regulation.

The CCAAT/enhancer binding protein (C/EBP) family of leucine zipper DNA binding (bZip) transcription factors control expression of a variety of genes involved in proliferation and differentiation in multiple tissues. Structurally, C/EBPs harbor highly conserved C-terminal basic DNA recognition and amphipathic leucine zipper dimerization domains and N-terminal transactivation domains. C/EBPβ is a member of this family and is involved in regulation of energy homeostasis (Tanaka et al., 1997; Yeh et al., 1995), immune functions (Screpanti et al., 1995; Tanaka et al., 1995), female reproduction (Sterneck et al., 1997), and tumorigenesis (Rask et al., 2000; Sundfeldt et al., 1999; Zahnow et al., 1997; Zhu et al., 2002).

C/EBPβ is regulated through extracellular cues including receptor-tyrosine-kinase (RTK) signaling and Ras-mediated MAPK pathway signaling that convert C/EBPβ from a suppressed and a repressive transcription factor into an active one (Kowenz-Leutz et al., 1994; Lamb et al., 2003; Nakajima et al., 1993). This occurs by abrogating the inhibitory function of a bipartite negative regulatory, repressive domain that is located between the N-terminal transactivation domain and the C-terminal bZip domain (Kowenz-Leutz et al., 1994; Williams et al., 1995).

An important group of transcription regulatory multiprotein complexes that interact with transcription factors has been termed "Mediator." Experiments in yeast revealed that Mediator transmits active and repressive information to regulate transcription (Boube et al., 2002). Mediator complexes consist of 20 to 30 protein subunits and display a size of about 2 megadaltons. The Mediator complex is thought to function as an integrator of signals and as a molecular bridge between DNA-bound transcription factors and the transcription apparatus including RNA polymerase II (RNAP II).

The yeast Mediator Gal11 subunit transmits the function of transactivators including Gal4 (Jeong et al., 2001), Swi5 (Bhoite et al., 2001), and Gcn4 (Swanson et al., 2003), or the Tup1 repressor (Han et al., 2001). The metazoan Mediator subunit that shares some homology with Gal11 is the CRSP130/DRIP130/TRIP150β/Sur2 protein (refereed to as CRSP130/Sur2) that is also a constituent of human and mouse Mediator complexes (Boube et al., 2002). The CRSP130/Sur2 protein was first identified as a downstream target of the Ras/MAP-kinase pathway in *Caenorhabditis elegans* (Singh and Han, 1995) and then as the major target of the conserved transactivation region 3 (CR3) of the adenoviral 13SE1A oncoprotein (Boyer et al., 1999). CRSP130/Sur2 was suggested to represent a major target of the MAPK pathway and to associate with transcriptional activators in a signal-dependent fashion (Stevens et al., 2002), suggesting that signal transduction could modify Mediator interactions via its CRSP130/Sur2 subunit.

Previously, C/EBPβ has been assigned cellular E1A functions (Spergel et al., 1992) and was found to be downstream of Ras-MAPK signaling (Kowenz-Leutz et al., 1994; Nakajima et al., 1993). Since CR3 of 13SE1A entirely suppressed transactivation by C/EBPβ (E.K.-L. and A.L., unpublished data), we explored the possibility that C/EBPβ functions are associated with Mediator interactions. Here we show that C/EBPβ directly interacts with Mediator complexes via the CRSP130/Sur2 subunit. Knocking down CRSP130/Sur2 expression or inhibiting CRSP130/Sur2 function by E1ACR3 prevents transcription activation by C/EBPβ. Activation of MAP-kinase signaling or upmutation of the MAP-kinase site in C/EBPβ alters its association with distinct Mediator

*Correspondence: aleutz@mdc-berlin.de

RESEARCH COMMUNICATION

Human CRSP interacts with RNA polymerase II CTD and adopts a specific CTD-bound conformation

Anders M. Näär,[1] Dylan J. Taatjes,[2] Weiguo Zhai,[2] Eva Nogales,[2,3] and Robert Tjian[2,4]

[1]Department of Cell Biology, Harvard Medical School, Massachusetts General Hospital Cancer Center, Charlestown, Massachusetts 02129, USA; [2]Howard Hughes Medical Institute and [3]Lawrence Berkeley National Laboratory, Department of Molecular and Cell Biology, Berkeley, California 94720, USA

Activation of gene transcription in mammalian cells requires several classes of coactivators that participate in different steps of the activation cascade. Using conventional and affinity chromatography, we have isolated a human coactivator complex that interacts directly with the C-terminal domain (CTD) of RNA polymerase II (Pol II). The CTD-binding complex is structurally and functionally indistinguishable from our previously isolated CRSP coactivator complex. The closely related, but transcriptionally inactive, ARC-L complex failed to interact with the CTD, indicating a significant biochemical difference between CRSP and ARC-L that may, in part, explain their functional divergence. Electron microscopy and three-dimensional single-particle reconstruction reveals a conformation for CTD–CRSP that is structurally distinct from unliganded CRSP or CRSP bound to SREBP-1a, but highly similar to CRSP bound to the VP16 activator. Together, our findings suggest that the human CRSP coactivator functions, at least in part, by mediating activator-dependent recruitment of RNA Pol II via the CTD.

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Regulation of eukaryotic RNA polymerase II (Pol II) transcription by sequence-specific enhancer and promoter-binding proteins is dependent on several different classes of cofactors and coactivators (Lemon and Tjian 2000; Malik and Roeder 2000; Peterson and Workman 2000; Näär 2001). Some of these coactivators are recruited to enhancer/promoter DNA by transcriptional activators to facilitate various steps in the gene activation process. For example, certain chromatin-directed activities, such as ATP-dependent nucleosome remodeling factors and histone acetyltransferases, assist enhancer and promoter-binding proteins and general transcription factors in accessing their target sequences in chromatin-packaged DNA (Lemon and Tjian 2000; Näär et al. 2001; Roth et al. 2001). Other classes of coregulators, such as TFIID, are more closely integrated with the transcriptional machinery and have been proposed to act at steps subsequent to chromatin remodeling to enhance activator-dependent recruitment of the transcriptional apparatus to the promoter (Albright and Tjian 2000; Näär et al. 2001). The TFIID complex, composed of TBP and associated TAFs, recognizes the TATA box and downstream promoter sequences and can be recruited to the promoter by activators.

A different class of cofactors, including yeast Mediator, do not directly bind promoter sequences, but can be recruited by activators. In addition, yeast Mediator can associate with RNA Pol II via the C-terminal domain (CTD) of the large RNA Pol II subunit and has been proposed to act as a bridge between activators and the transcriptional machinery (Myers and Kornberg 2000). The RNA Pol II CTD is composed largely of tandem repeats of the YSPTSPS consensus amino acid sequence; yeast CTD contains 26 repeats, whereas mammalian CTD harbors 52 repeats. The CTD appears to serve multiple functions in the transcription initiation and elongation process. Recently, the CTD has also been shown to play a role in coupling gene transcription to mRNA processing events, such as 5′-capping, splicing, RNA cleavage, and polyadenylation (Gerber et al. 1995; Cho et al. 1997; Corden and Patturajan 1997; McCracken et al. 1997a,b; Tanner et al. 1997; Yue et al. 1997; Hirose et al. 1999; Otero et al. 1999; Conaway et al. 2000).

A family of human cofactor complexes distantly related to yeast Mediator have been isolated recently (Fondell et al. 1996; Jiang et al. 1998; Sun et al. 1998; Boyer et al. 1999; Gu et al. 1999; Näär et al. 1999; Rachez et al. 1999; Ryu et al. 1999). Unlike yeast Mediator, however, these human cofactor complexes (which include ARC/DRIP, TRAP/SMCC, NAT, CRSP, and PC2) have not been shown to interact with the CTD of RNA Pol II.

In a recent study, we discovered that the human activator recruited cofactor fraction (ARC) consists of two distinct complexes, ARC-L and CRSP (Taatjes et al. 2002). Both are highly related, but display contrasting cofactor properties. ARC-L is somewhat larger and contains additional subunits (ARC240, ARC250, cdk8, cyclin C) not present in CRSP, whereas CRSP contains a 70-kD subunit (CRSP70) not present in ARC-L. On the basis of subunit composition and in vitro transcription assays, ARC-L most closely resembles the NAT and SMCC cofactor complexes (Sun et al. 1998; Gu et al. 1999; Taatjes et al. 2002). Previous studies with NAT, SMCC, and ARC/DRIP revealed weak interactions with RNA Pol II, but direct and specific binding to the CTD was not observed (Sun et al. 1998; Gu et al. 1999; Näär et al. 1999; Chiba et al. 2000). Here, we show that the human CRSP coactivator complex, but not ARC-L, interacts strongly with the CTD of RNA Pol II. CTD-affinity chromatography specifically isolated a large, multisubunit complex indistinguishable from the previously identified CRSP coactivator. Both complexes possess highly similar or identical subunit composition and display indistinguishable coactivator function in vitro. Further, structural analysis of the CTD-binding complex by electron microscopy (EM) and single particle reconstruc-

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[4]Corresponding author.
E-MAIL jmlim@uclink4.Berkeley.edu; FAX (510) 643-9547.
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Isw1 Chromatin Remodeling ATPase Coordinates Transcription Elongation and Termination by RNA Polymerase II

Antonin Morillon,[1] Nickoletta Karabetsou,[1] Justin O'Sullivan,[2] Nicholas Kent,[1] Nicholas Proudfoot,[2] and Jane Mellor[1,*]
[1]Department of Biochemistry
University of Oxford
South Parks Road
Oxford OX1 3QU
United Kingdom
[2]Sir William Dunn School of Pathology
University of Oxford
South Parks Road
Oxford OX1 3RE
United Kingdom

Summary

We demonstrate that distinct forms of the yeast chromatin-remodeling enzyme Isw1p sequentially regulate each stage of the transcription cycle. The Isw1a complex (Iswlp/Ioc3p) represses gene expression at initiation through specific positioning of a promoter proximal dinucleosome, whereas the Isw1b complex (Iswlp/Ioc2p/Ioc4p) acts within coding regions to control the amount of RNA polymerase (RNAPII) released into productive elongation and to coordinate elongation with termination and pre-mRNA processing. These effects of Isw1b are controlled via phosphorylation of the heptad repeat carboxy-terminal domain (CTD) of RNAPII and methylation of the chromatin template. The transcription elongation factor Spt4p antagonizes Isw1p and overcomes the Isw1p dependent pausing of RNAPII at the onset of the elongation cycle. Overall these studies establish the central role played by Isw1p in the coordination of transcription.

Introduction

Chromatin plays an important role in transcriptional regulation and is generally considered to impede transcription initiation and elongation by RNA polymerase II (RNAPII). Chromatin structures over promoters are regulated by enzymes that covalently modify histones (Strahl and Allis, 2000) or alter chromatin by ATP-dependent disruption of DNA-histone interactions (Becker and Horz, 2002). These proteins are generally recruited to promoters by sequence-specific DNA binding proteins (Cosma et al., 1999). During the elongation phase of transcription, RNAPII also uses a wide range of accessory factors to facilitate its movement through chromatin (Hartzog, 2003) and several RNAPII-associated complexes have been identified (Shilatifard et al., 2003). For example, in yeast, some complexes, such as PAF (Krogan et al., 2002), Spt4/5, and TFIIS (Dst1p) (Pokholok et al., 2002) are all associated with RNAPII throughout the elongation phase. However, other factors are localized to the 5′ region of genes such as capping enzymes (Cho et al., 1997, 1998; McCracken et al., 1997; Rodriguez et al., 2000) or to the 3′ region of genes such as the cleavage/polyadenylation complex CF1A (Licatalosi et al., 2002). Differential association between complexes and RNAPII appears to be a function of the heptapeptide repeat (Tyr-Ser-Pro-Thr-Ser-Pro-Ser) (Allison et al., 1985) of the carboxy-terminal domain (CTD) of the largest subunit, Rpb1p, which is subject to differential phosphorylation. Hyperphosphorylation at Ser5 of the CTD by the Kin28p kinase subunit of TFIIH promotes disengagement of the enzyme from the promoter into the elongation phase of transcription (Rodriguez et al., 2000) coupled with the exchange of initiation factors for elongation factors (Pokholok et al., 2002). Ser5 phosphorylation then diminishes in the coding region while levels of Ser2 phosphorylation increase as RNAPII moves toward the 3′ region (Komarnitsky et al., 2000). Significantly capping factors (Cho et al., 1998; McCracken et al., 1997; Rodriguez et al., 2000; Schroeder et al., 2000) and the Set1p complex (Ng et al., 2003) interact with phospho-Ser5 CTD while CF1A (Barilla et al., 2001; Licatalosi et al. 2002), and the Set2p histone methylase (Krogan et al., 2003; Li et al., 2003; Xiao et al., 2003), interact with phospho-Ser2 CTD. Thus, the differential association of complexes to the CTD links the various phases of pre-mRNA synthesis, processing, and export. Furthermore, differential patterns of histone H3 methylation by Set1p (H3-K4) and Set2p (H3-K36) may respectively mark out the early and later stages of elongation and promote association of additional factors to the chromatin template to regulate these events (Krogan et al., 2003; Ng et al., 2003; Xiao et al., 2003). Candidates for this function are the FACT complex that facilitates transcription through chromatin (Orphanides et al., 1999), and the chromodomain containing chromatin-remodeling ATPase, Chd1p (Woodage et al., 1997), recently proposed to function as an elongation factor (Simic et al., 2003). Chd1p has also been identified as a termination factor at some yeast genes but at other genes Chd1p functions redundantly with the imitation switch (ISWI) chromatin-remodeling ATPases, Isw1p and Isw2p (Alen et al., 2002). Furthermore, we have previously observed a highly localized chromatin organization within the coding regions of a number of yeast genes that is dependent on the catalytic activity of Isw1p chromatin remodeling activity (Kent et al., 2001). We therefore address in this study the potential role played by Isw1p in the elongation and termination of transcription.

Chromatin remodeling enzymes of the ISWI type (Clapier et al., 2001) are widely found in eukaryotes and are implicated in events leading to repression of expression. Thus, ISWI is associated with nontranscribed regions of polytene chromosomes in *Drosophila* (Deuring et al., 2000) and may displace TBP from promoters in *Xenopus* (Kikyo et al., 2000) and yeast (Moreau et al., 2003). However, a positive role for ISWI cannot be excluded as it is required for the expression of some genes in *Drosophila* (Badenhorst et al., 2002). In addition, microarray data in yeast show that Isw1p and Isw2p, the two ISWI homo-

*Correspondence: jane.mellor@bioch.ox.ac.uk