Instituto Juan March
de Estudios e Investigaciones

112 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

The Myc Network: Regulation of Cell Proliferation, Differentiation and Death

Organized by

R. N. Eisenman and J. León

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D. E. Ayer
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Introduction

R. N. Eisenman and J. León

Over the last decade there has been a virtual explosion of information on the Myc network. In part this is simply an outgrowth of the longstanding interest in Myc stemming from its involvement in many types of cancer and the fundamental cellular processes of proliferation, differentiation, growth, and apoptosis. However, when Myc was shown to heterodimerize with Max and function as a DNA-binding transcription factor in 1990-1992, the interest of many laboratories became focused on attempting to understand how Myc's role in transcription directly relates to its biological functions in normal and cancer cells. To a large extent this has involved determining the molecular mechanism(s) whereby Myc regulates gene expression and, very importantly, the specific genes that are targets of Myc. The biology of Myc has also come under intense scrutiny as the role of Myc in cell cycle regulation and the importance of Myc-induced apoptosis in oncogenesis have become more apparent. Furthermore targeted deletions of Myc genes in mice and in tissue culture cells as well as genetic analysis of Myc function in Drosophila has highlighted roles for Myc in development, cell cycle, and cell growth.

In addition to the intense interest in Myc itself, the field has also expanded in other, initially unexpected, directions. This has come from the realization that Max, while an obligate dimerization partner for Myc, also interacts with other proteins (Mad, Mnt, Mga) and these proteins, in turn, appear to influence the functions of Myc. For example, much work on the Mad protein family suggests that Mad proteins act at least in part to antagonize Myc function and are likely to be involved in cell cycle exit and differentiation. Furthermore, work on understanding the repression function of Mad has led to identification of novel co-repressors (mSin3) and an analysis of the role of chromatin modification in repression in general. New evidence suggests that Myc interacts with a co-activator and may also influence chromatin structure. Another layer of complexity may arise from the findings that other novel proteins, such as Mlx, may potentially serve to connect specific members of the Myc network to other transcription factor modules.

The idea of the Myc network grew out of the increasing realization that Myc does not function alone but within an immediate context of interacting proteins as well as within a specific cellular environment. The goal of this workshop was to draw together what has become a rather diverse field of research in what is first international meeting entirely dedicated to the Myc network. The workshop dealt with the most recent advances in this active field of research, from the molecular level to the most complex biological models. The talks and posters included the presentation of the molecular structure of Myc:Max , Mad:Max, and Mad:Sin3 complexes;

new Max homologs, the Drosophila Mad orthologs, the interaction of Myc with proteins involved in chromatin remodeling and transcriptional regulation, new Myc target genes which are either positively or negatively regulated by
Myc, insights to regulation of Myc expression, Myc interactions in tumorigenesis and, finally, new data on Myc functions in differentiation, apoptosis and cell growth, using Drosophila and mice as model systems.

While many questions on Myc functions and Myc target genes are still to be resolved, the workshop succeeded in enriching our view of the problems and setting the scenario for future developments. Some of the key issues that arose at the workshop were as follows:

1. Mechanisms of Myc activation and repression and the relative importance of activation vs. repression in Myc function.
2. Functional differences between normal and deregulated Myc expression/overexpression.
3. Criteria for defining Myc and Mad target genes and strategies for relating target gene function to Myc and Mad biological effects.
4. Relationship between Myc's roles in cell proliferation, growth, and apoptosis.

We would like to thank the speakers for their presentations and for open and comprehensive discussions. We are also grateful to Juan March Foundation for their excellent support and organization of the workshop.

Robert N. Eisenman and Javier León

Session 1: Network Interactions and Transcriptional Mechanisms
Chair: Gerard Evan

Essential cofactors for Myc-mediated oncogenesis

Michael Cole

Department of Molecular Biology
Princeton University, Princeton, NJ 08544

Functional studies demonstrate that the oncogenic activity of the c-Myc protein requires a small 20 amino acid segment from the N-terminus (MBII) that is conserved in all members of the *myc* family of genes. This observation provided an avenue to study the function Myc through an analysis of the nuclear proteins that bind through MBII and which are also essential for the transforming activity of the oncogene. The experimental approach was based on the observation that dominant interfering alleles of the c-Myc protein form protein complexes that are dependent on the integrity of MBII and hence correlate with nuclear factors that may be essential for c-Myc function (Brough et al., 1995).

Biochemical purification of c-Myc associated cofactors initially led to the identification of the 430 kD cofactor TRRAP (TRansactivation/tRansformation domain Associated Protein (McMahon et al., 1998). The TRRAP nuclear cofactor is distantly related to the PI3 kinase/ATM family and essential for oncogenic transformation by two different oncoprotein networks, the Myc family and the E2F family. We have extended this work to show that TRRAP interacts with the N-Myc and L-Myc oncoproteins (manuscript in preparation). The *S. cerevisiae* homologue of human TRRAP was an uncharacterized hypothetical ORF which had never been identified in genetic screens for mutations in cell cycle or gene regulation pathways. We deleted the gene by homologous recombination and showed that the yeast TRRAP homolog (TRA1) is essential for viability. The TRRAP(TRA1p) protein is evolutionarily more ancient than Myc, since there is no c-Myc homologue in yeast, and therefore Myc presumably evolved to recruit TRRAP to specific genomic sites. More importantly, TRA1p was shown to be a component of the SAGA complex (Saleh et al., 1998), providing a link between Myc and the histone acetyltransferase GCN5 and other proteins implicated in chromatin remodeling.

Based on the findings in yeast, we showed that c-Myc can recruit hGCN5 and an associated HAT activity *in vivo* (McMahon et al., 2000). Furthermore, TRRAP and hGCN5 exist in a complex independent of c-Myc, indicating that TRRAP is the intermediary for the recruitment of hGCN5 by c-Myc. Since c-Myc function is inhibited by recruitment of histone deacetylase activity through Mad family proteins, these opposing biochemical activities may be responsible for the antagonistic biological effects of c-Myc and Mad on target genes and ultimately on cellular transformation.

TRRAP was only the first of several cofactors we identified by affinity purification with the c-Myc transactivation domain. Two other proteins were identified as TIP49 and a novel related protein that we called TIP48, both of which are highly conserved in evolution and contain ATPase/helicase motifs (Wood et al., 2000). TIP49 and TIP48 are complexed with c-Myc *in vivo* and binding is dependent on the MBII domain that is essential for oncogenic activity. To establish a functional role in Myc-mediated transformation, we showed that a missense mutation in the TIP49 ATPase motif acts as a dominant inhibitor of c-Myc oncogenic activity but does not inhibit normal cell growth. The same dominant negative

TIP49 protein also inhibits E1A- mediated transformation and binds to E2F-1 (Dugan et al., manuscript in preparation). The TIP49 and TIP48 ATPase/helicase proteins represent a novel class of cofactors recruited by transcriptional activation domains that function in diverse pathways.

One other protein that tightly associates with c-Myc *in vitro* and coprecipitates *in vivo* is the BAF53 actin-related protein ((Zhao et al., 1998); Wood et al., manuscript in preparation). Actin-related proteins (ARPs) have been found in several chromatin remodeling complexes throughout evolution, including Swi/Snf and RSC. The most intriguing aspect of BAF53 binding to c-Myc is that we find no binding of the Brg/Brm ATPase/helicase-related subunits of the mammalian Swi/Snf complex. Mutant forms of BAF53 inhibit c-Myc mediated focus formation in cooperation with the H-ras(G12V) oncogene, providing evidence for a functional role for ARPs in oncogenic transformation.

The purification of unique nuclear cofactors that interact directly with the Myc family of proteins has offered important insights into the mechanism of oncogenic transformation. Ongoing studies are aimed at understanding the role of individual cofactors in the different Myc biological activities such as normal cell proliferation, apoptosis, blocking differentiation, and the regulation of specific chromosomal target genes.

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Development of molecular models for the action of c-Myc and N-Myc using a chromatin immunoprecipitation assay

Peggy Farnham

University of Wisconsin Medical School, Madison, WI 53706

Although it is clear that overexpression of Myc family members can lead to neoplastic transformation, the relative importance of Myc as a transcriptional regulator in mediating neoplastic transformation is highly debated. Much of this controversy comes from the difficulty in clearly defining a true Myc target gene (1). Since other proteins (such as USF) that can bind to the same DNA element as the Myc/Max heterodimer also exist in cells, it has been difficult to classify cellular promoters as being activated specifically by Myc. To address the question of target gene specificity, we have used a formaldehyde crosslinking and chromatin immunoprecipitation procedure to examine binding of c-Myc and N-Myc to cellular promoters.

We have demonstrated that transcription from the mouse, hamster, and human cad promoters is growth responsive and the growth responsive element maps to a consensus E box located 65 bp downstream of the transcription start site (2,3,4). To determine if the c-Myc protein is a critical mediator of the cell cycle stage-specific expression of the cad gene, we used a chromatin immunoprecipiation procedure to monitor binding of Myc to the endogenous cad promoter after quiescent cells were stimulated to reenter the cell cycle (4). We found that both USF and Myc bound to the cad promoter. However, the amount of Myc bound to the cad promoter increased 8 fold as cells moved from G0 to S phase whereas USF binding was constitutive. Because the cad promoter was transcriptionally active in S phase when both Myc and USF were bound but was inactive in quiescent cells when only USF bound, this suggested that the S phase transcription of the cad promoter may be due to Myc. However, other possibilities, such as an S phase–specific modification of USF could not be ruled out. To determine if Myc was indeed the regulator of cad transcription, we performed two additional experiments. First, we replaced the E box with a Gal4 binding site and showed that Gal4Myc, but not Gal4USF, could activate the cad promoter. Second, we created stable cell lines harboring cad promoter constructs having a wt or an altered E box or lacking an E box. The wt promoter bound both USF and Myc whereas the construct lacking an E box did not bind either USF or Myc, demonstrating that the same sequence was needed for the binding of the two proteins. The altered E box differed from the wt in the nucleotides flanking the CACGTG (5). Using the chromatin immunoprecipitation assay, we found that this altered E box bound USF but no longer could be recognized by Myc. Analysis of promoter activity demonstrated that the cad promoter having the altered E box did not display S phase-specific transcription (6). Taken together these results suggest that cad is a true Myc target gene.

The *N-myc* gene is amplified in many human neuroblastomas and this amplification serves as a poor prognostic factor. However, few genes have been determined to be direct targets of N-Myc. To identify N-Myc target genes, we performed differential expression screens with neuroblastoma cells containing high versus low levels of N-Myc (7). We identified twenty-two genes upregulated by N-Myc and one gene downregulated by N-Myc. However, only 5 of these genes responded to increased N-Myc levels in more than one system. We utilized a formaldehyde crosslinking and immunoprecipitation procedure to

determine if N-Myc was bound to the promoters of these putative target genes in living cells. We found that low levels of N-Myc were bound to the promoters of the telomerase and prothymosin genes in neuroblastoma cells having normal levels of N-Myc but that the amounts of N-Myc bound to these promoters greatly increased upon overexpression of N-Myc. However, the amount of Max bound to the promoters was high before and after induction of N-Myc. Therefore, our studies suggest that N-Myc competes with other Max partners for binding to target promoters. Interestingly, previous studies have shown a threshold effect for N-Myc amplification in human tumors. Our use of the chromatin immunoprecipitation assay has provided a molecular explanation for these clinical observations.

Finally, we have begun investigation into the mechanisms by which recruitment of c-Myc or N-Myc to target promoters leads to transcriptional activation. Others have shown that Myc can bind to histone acetylases and have suggested that Myc mediates transcriptional activation by causing an increase in the levels of acetylated histones on target promoters. To directly test this model, we again employed the chromatin immunoprecipitation assay to examine the levels of acetylated histones on the cad promoter (8). We found high levels of acetylated histones on the cad promoter in both G0 and S phase. We also examined acetylated histones on the cad promoter during and after differentiation of U937 cells. Although the levels of Myc bound to the cad promoter were greatly reduced after differentiation, we saw high levels of acetylated histones on the cad promoter both before and after differentiation. Thus, the recruitment of Myc to a target promoter does not influence the amount of acetylated histones at that promoter. In contrast to the cad promoter region, we found very low levels of acetylated histones on the 3' end of the cad gene and also found very low levels on the inactive albumin promoter. Thus, our results suggest that histone acetylation can identify a promoter region which has transcriptional potential in a certain cell type, but does not distinguish between inactive vs active states of that promoter.

In summary, we have shown that recruitment of Myc family members correlates with the transcriptional regulation of certain cellular genes in both normal and cancer cells. However, the mechanism by which Myc mediates transcriptional regulation does not appear to involve changes in histone acetylation. Further investigations are in progress to define the role of Myc in transcriptional activation.

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Mondo, a novel bHLHZip transcriptional activator that interacts genetically with Myc

Andrew N. Billin, Alanna L. Eilers, Jennifer S. Logan, and Donald E. Ayer

Huntsman Cancer Institute, University of Utah, Salt Lake City, Utah 84112-5550. E-mail: don.ayer@hci.utah.edu

The Mad family of transcriptional repressors, Mad1, Mxi1, Mad3, Mad4, are antagonists of Myc and appear to regulate cell cycle exit during diverse cellular differentiation programs. In a number of different *in vitro* assays, members of the Mad family appear to function identically. For example, all heterodimerize with Max and repress transcription by targeting the mSin3A/HDAC corepressor complex to CACGTG E-box-containing promoters. Furthermore, all Mad family proteins function as potent antagonists of Myc + Ras cotransformation. However the expression profiles of the different Mad family members and the phenotypes of Mad1 and Mxi1 null mice suggest that the functions of different Mad family members are not completely redundant. To explore differences in Mad family function we have identified and characterized a new Mad binding protein termed Mlx for Max-like-protein-X.

Mlx a member of the bHLHZip class of transcription regulators and database searches revealed that is most highly related to Max. Our analysis suggests that Mlx and Max are also functionally related. The similarities between Mlx and Max include: 1) Broad expression in many tissues, 2) Long protein half-life and 3) Formation of heterodimers with Mad family proteins that are capable of specific CACGTG binding. We show that transcriptional repression by Mad1:Mlx heterodimers is dependent on dimerization, DNA binding, and recruitment of the mSin3A/HDAC corepressor complex. In contrast with Max, Mlx interacts only with Mad1 and Mad4. Together, these findings suggest that Mlx may act to diversify Mad family function by its restricted association with a subset of the Mad family of transcriptional repressors.

The structural and functional similarity to Max, suggested that Mlx might have multiple protein partners. Using full-length Mlx as bait in a two-hybrid screen we identified 40 interacting clones. One clone encoded Mad4 and the other 39 encoded a novel bHLHZip protein that defines a new family of transcription factors. Due to the large size of the mRNA encoding the prototypic member of this family, we have termed these new transcription factors the Mondo family. We have identified two closely related paralogs in human cells MondoA and MondoB, and homologs in both *C. elegans* and *D. melanogaster*. MondoA preferentially forms heterodimers with Mlx and this heterocomplex can bind to, and activate transcription from, CACGTG E-boxes. MondoA:Mlx heterocomplexes localize predominantly to the cytoplasm of cultured mammalian cells, but activate transcription when targeted to the nucleus. The amino terminus of the Mondo proteins is highly conserved among family members and regulates both cytoplasmic localization and transactivation of MondoA:Mlx heterodimers. To understand the function of the Mondo family, we identified mutations in the *dmondo* locus. Flies homozygous for the *dmondo* mutation are subviable demonstrating that *dmondo* is required for normal development. Given the functional similarities between Myc and MondoA, we tested for genetic interaction between *dmyc* and

dmondo. These experiments demonstrated a synthetic lethal relationship between *dmondo* and *dmyc*, suggesting that they act in the same or overlapping genetic pathways. Finally, *dmondo* gene expression is positively regulated by *dmyc* suggesting a mechanism for their genetic interaction.

In conclusion, we have described a new transcription factor network whose center is Mlx. Like Max, Mlx is a common partner for transcriptional activators, the Mondo proteins, and repressors, the Mad proteins. Unexpectedly, the Max and Mlx networks interact genetically. The expression of the *dmondo* gene is regulated by *dmyc*, suggesting that the basis for this genetic interaction is transcriptional crosstalk. Thus, a web of regulatory interactions between the Max and Mlx networks is likely to regulate the function of these two transcription factor families. Future experiments will be directed at understanding the complicated interplay of these two networks.

Identification of novel partners of the ROX/Mnt bHLHZip transcription factor

Germana Meroni and Alexandre Reymond

Telethon Institute of Genetics and Medicine, Milan, Italy

The Myc proto-oncogene members encode central regulators of cell proliferation, differentiation and apoptosis. They associate with the bHLHZip protein Max to bind specific DNA sequences and regulate the expression of genes important for growth and cell cycle progression. The other family members Mad1, Mxi1, Mad3, Mad4, Mga and Rox/Mnt antagonize their activities. The Mads, Mga and Rox/Mnt compete with Myc in heterodimerizing with Max and in binding to some of its specific target sequences. The Mads:Max and Rox:Max dimers repress transcription through binding to the mSIN3 corepressor protein and by tethering histone deacetylase-containing complexes to the DNA.

The ability of Rox/Mnt to activate transcription in some cell lines; its subcellular distribution, peculiarly observed in spots within the nucleus; and the presence of large portions of the protein sharing no homology with the Mads, suggest the idea of a more complex Rox/Mnt function with respect to the other Myc antagonists (Meroni et al., 1997; Hurlin et al., 1997). This prompted us to move towards the study of possible additional Rox/Mnt partners.

In a screen for Rox interactors we isolated Mlx, a new Max-like bHLHZip family member (Meroni et al., *in press*). The same protein has been independently identified in a screening with Mad1 (Billin at al., 1999). We found that through its bHLHZip domain, Mlx is able to homodimerize and to heterodimerize with Rox. These dimers bind DNA at the level of the canonical and the CACGCG non-canonical E-box sequences and, while the homodimers appear to be inert when tested in transactivation assays, the Mlx-Rox dimer repress transcription. We identified three different isoforms of Mlx that behave in the same way with respect to the dimerization and DNA binding properties but differs in subcellular distribution one being nuclear and the other two both nuclear and cytoplasmic. All these features, together with the fact that Mlx is the only member of the family that is not able to heterodimerize with Max, candidates Mlx to play a central role in a novel network of E-box binding complexes.

In support of this hypothesis, we have found that Mlx is able to heterodimerize with two new bHLHZip proteins, Mio and Mir. These heterodimers function as transcriptional repressors or transcriptional activators showing that this network, like the Max network, will have positive and negative constituents. Interestingly the newly identified bHLHZip proteins, Mio and Mir, display a similar restricted pattern of expression during organogenesis suggesting a possible role of these proteins in development. We are currently studying the function of this emerging transcription factors regulatory pathway in cell growth and differentiation.

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Structural basis for heterodimerization and DNA recognition by Myc-Max and Mad-Max

Satish K. Nair * and Stephen K. Burley

Rockefeller University and Howard Hughes Medical Institute
New York, NY 10021

The oncogenic potential of the c-myc oncogene is predicated upon heterodimerization of the c-Myc protein with Max prior to recognition of a cognate hexanucleotide element (the E-box) and transcriptional activation (1). In contrast, transcriptional repression, and subsequent cellular proliferation, results from the formation of a complex between Max and Mad and the recognition of the identical E-box hexanucleotide by the Mad-Max heterodimer (2). Heterodimerization and sequence-specific DNA recognition by Myc-Max and Mad-Max are solely functions of their basic/helix-loop-helix/leucine zipper (bHLHZ) domains (3). In order to understand the molecular bases for heterodimerization and sequence-specific DNA recognition by these heterodimers, we have determined the high-resolution crystal structures of Myc-Max and Mad-Max bHLHZ domains bound to near identical oligonucleotides bearing their cognate recognition elements. Using novel, stereoselective chemical ligation techniques (4), we have been able to produce directionally ligated heterodimers, thus averting the unwanted formation of homodimers which may hinder crystallographic efforts. High resolution electrospray mass spectometric methods document that the purified samples are composed entirely of the appropriate heterodimeric species. Co-crystal structures of Myc-Max and Mad-Max, respectively bound to 19 and 18 base pair oligonucleotide bearing the E-box element, have been determined to resolution of 1.8 and 2.0 Å, respectively. These high resolution crystal structures, in addition to the previously determined structure of the Max homodimer (5), provide a understanding of the structural and stereochemical basis for heterodimerization and sequence-specific DNA recognition. We complement these structures with mutational and hydrodynamic studies. We are also developing methodologies for a structure-based high-throughput screen to document properties of the target oligonucleotide sites necessary for specificity (6,7).

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Session 2: Myc/Mad in Cell Cycle and Apoptosis
Chair: Michael Cole

Repression of p15ink4b expression by Myc via association with Miz-1

Peter Staller, Karen Peukert, Joan Seoane1), Jiri Lukas2), Holger Karsunky3), Tarik Möröy3), Jiri Bartek2), Joan Massague1) , Frank Hänel4) and Martin Eilers

Institute of Molecular Biology and Tumour Research, Marburg, 1)Cell Biology Program; Memorial Sloan-Kettering Cancer Center, New York, , 2)Institute of Cancer Biology, Copenhagen 3) Institute for Cell Biology, University of Essen, §Hans-Knöll-Institut für Naturstoff-Forschung, Jena.

Deregulated expression of *c-myc* can induce cell proliferation in established cell lines, prevent senescence of primary mouse embryo fibroblasts (MEFs) and render cells resistant to antiproliferative factors like TGF-β. Both transcriptional activation and repression by Myc have been implicated in these effects. While activation by Myc is mediated by interaction with Max, the molecular interaction(s) via which Myc represses transcription of specific genes have not been resolved. We have found that a Myc-associated transcription factor, Miz-1, induces G1 arrest in a pocket protein-dependent manner and inhibits Cyclin D-associated kinase activity. Miz-1 upregulates expression of p15ink4b by binding to the Initiator element of the p15ink4b promoter. Myc forms a ternary complex with Miz-1 at the p15 Initiator, preventing induction by Miz-1. Expression of Myc in primary MEFs inhibits the accumulation of p15ink4b associated with cellular senescence; conversely, deletion of Myc in an established cell line activates p15ink4b expression. Alleles of Myc that are unable to bind to Miz-1 fail to inhibit accumulation of p15ink4b mRNA in primary cells. As a consequence, they are deficient in immortalization showing that inactivation of Miz-1 function is critical for immortalization by Myc.

CycD1 and CycD2 are required for Myc-induced proliferation but dispensable for apoptosis

Ignacio Pérez-Roger* and Hartmut Land
Imperial Cancer Research Fund. 44 Lincoln's Inn Fields, London WC2A 3PX, UK. *Present Address: Instituto de Biomedicina de Valencia (CSIC). Jaime Roig 11, 46010-Valencia, Spain

CycE/Cdk2 kinase activation is an essential step in Myc-induced proliferation, which requires sequestration of G1 cell cycle inhibitors p27Kip1 and p21Cip1 (1, 2, 3). D-type cyclins are thought to interfere with the activity of p27Kip1 and p21Cip1 in their inhibition of CycE/Cdk2 complexes (4, 5). Here we show direct genetic evidence for the involvement of cyclins D1 and D2 in Myc-induced proliferation. Using primary knock-out mouse embryo fibroblasts we show that cells lacking either CycD1 or CycD2 are unable to respond to Myc with increased proliferation but they undergo accelerated cell death in the absence of serum. The response to Myc in cyclin D1 knock out cells can be restored by constitutive expression of CycD1, CycD2 or a CycD1 mutant able to bind CDKs and CKIs but unable to stimulate kinase activity (6). This shows that the link between the D-type cyclins and Myc-induced proliferation is independent of CycD/Cdk activity but instead it correlates with the ability of CycD/Cdk complexes to sequester CKIs. The sequestration function of the D-type cyclins thus appears essential for Myc-induced cell cycle progression but dispensable for apoptosis.

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c-Myc antagonizes p53 in human leukemia cells

Javier León

Dpto. de Biología Molecular, Facultad de Medicina, 39011 Santander, Spain

Overexpression of c-*myc* is found in many solid tumors and in some leukemias. In chronic myeloid leukemia (CML), the disease progresses from a benign chronic phase to a blast crisis phase. This progression is frequently accompanied by overexpression of c-Myc, while p53 remains in wild-type in the majority of the cases. We used the CML-derived K562 cell line as a model to study c-Myc role in differentiation and p53 functional interactions. These cells can be differentiated *in vitro* into distinct myeloid lineages. By using K562 sublines with conditional expression of Myc and Max we showed that the in vitro differentiation of K562 into erythroid lineage was impaired by c-Myc and promoted by Max, while the megakaryocytic differentiation was unaffected. In CML Ras mutation is practically absent, and consistently, in K562, Ras oncoproteins inhibit growth and induces $p21^{WAF1}$ expression through a p53, $p16^{INKa}$ and $p14^{ARF}$-independent mechanism. In these cells, Myc expression inhibits Ras signaling as assessed by abrogation of the Ras/MAPK-mediated transactivation the proximal $p21^{WAF1}$ and collagenase (AP1-containing) promoters.

To study Myc-p53 functional interference we have generated double K562 transfectant cell lines with conditional expression of either c-Myc or p53. The cells expressed the $p53^{Val135}$ mutant, which adopts a wild-type conformation at 32°C, while c-Myc induction was achieved with a zinc-inducible expression vector. We found that p53 in wild-type conformation resulted in growth arrest and apoptosis of K562, as well as endogenous c-Myc down-regulation. However, when co-expressed, c-Myc partially rescued the cells from p53-mediated apoptosis. This apoptosis inhibition was assessed by cell morphology, annexin V binding, metabolic activity and DNA laddering. Myc did not interfere with p53 localization or induced its degradation. However, the expression of Myc inhibited p53-mediated transactivation of several p53-responsive promoters as Mdm2, Bax, $p21^{WAF1}$, PG13 –but not cyclin G. Shift to 32°C was accompanied by down-regulation of cyclinE/Cdk2 kinase activity and Rb hypophosphorylation, and both effects were counteracted by c-Myc.

The data are consistent with the c-Myc overexpression in the presence of wild-type p53 observed in most CML blast crisis cases. Moreover, c-Myc is capable of inhibiting p53-mediated transactivation on cell lines other than K562. These include other CML cell lines as well as prostate cancer, Burkitt lymphoma, acute myeloid leukemia and lung epithelia cell lines. These results open the hypothesis that c-Myc overexpression may antagonize the pro-apoptotic or anti-proliferative functions of p53, thus providing a molecular mechanism for the frequently observed deregulation of c-*myc* in human tumors.

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Myc and the ARF/p53/Mdm2 tumor suppressor pathway

Martine F. Roussel[1], Frederique Zindy[1], Christine M. Eischen[2], Jason D. Weber[1,3], John L. Cleveland[2] and Charles J. Sherr[1,3]. Departments of Tumor Cell Biology [1], Biochemistry [2] and the Howard Hughes Medical Institute [3], St. Jude Children's Research Hospital, Memphis, TN, 38105, USA.

The Myc oncogene is often found constitutively overexpressed in many cancers. In most somatic cells, c-Myc functions are necessary for the normal rate of progression of quiescent cells into the DNA synthetic (S) phase. Thus, in tumor cells, enforced c-Myc expression can confer a proliferative advantage by providing constitutive growth signals. Paradoxically, Myc activation can, under limiting growth conditions, initiate an endogenous apoptotic program (Evans). Therefore, Myc overexpression triggers a potent tumor surveillance response that effectively opposes hyperproliferation by killing those cells in which Myc levels exceed a safe threshold.

In mouse embryo fibroblasts (MEFs), the ARF/p53/Mdm2 pathway mediates Myc-induced apoptosis. $p19^{ARF}$, the product of the mouse INK4a/ARF locus regulates the function of the p53 transcription factor by binding to the p53 negative regulator Mdm2, inhibiting its E3 ubiquitin ligase activity and sequestering it into the nucleolus, thereby enabling p53 to accumulate in the cytoplasm where it induces genes that trigger apoptosis or growth arrest. ARF-null mice are highly tumor prone and ARF-null MEFs like p53-null MEFs, grow as established cell lines, do not undergo replicative senescence and can be transformed by oncogenic ras alone. This is in contrast to primary MEFs, which require co-transformation by an immortalizing gene such as Myc or E1A and led us to hypothesize that deletion of ARF might mimic MYC or E1A function.

Indeed, we showed that in wild type MEFs Myc activation rapidly elevates the levels of ARF and p53 and induces apoptosis. However, the few cells that survive Myc killing exhibited either p53 mutations (75% of cases) or biallelic ARF loss (25% of cases), facilitating Myc's ability to immortalize these cells. MEFs lacking ARF or p53 function are resistant to Myc-induced apoptosis and in these cells, Myc acts as a pure growth promoter.

Several oncogenes, including Myc, E1A, E2F-1, v-Abl and RasV12 have now be shown to trigger a p53-dependent oncogene checkpoint gated by ARF, which represents a fail-safe mechanism against excess proliferative signals. We tested this general concept *in vivo* by using the Eμ-Myc transgenic mouse in which Myc is overexpressed in B-cell progenitors under the control of the immunoglobulin heavy chain enhancer. After a protracted subclinical course during which the increase rate in B cell division is offset by a high apoptotic index, Eμ-Myc mice develop clonal pre-B and B-cell lymphomas similar to those observed in Burkitt's lymphomas bearing a translocated (t8; 14) c-Myc allele. We found that c-Myc-induced lymphomagenesis in Eμ-Myc mice selectively targeted the ARF/p53/Mdm2 pathway. Analysis of B-cell tumors revealed inactivation of p53 (in 25% of cases) or ARF (in an equal number of cases 25%), or overexpression of Mdm2 (in 25 % of cases). In some tumors, Mdm2 was overexpressed in the absence of p53, suggesting that Mdm2 can induce tumors by interacting with other targets. As expected, Eμ-Myc mice hemizygous for ARF displayed accelerated disease (about 3 months mean survival) and all tumors lost the wild type ARF allele. All ARF-null Eμ-Myc mice died

of lymphoma within a few weeks of birth. About half of the tumors arising in ARF-hemizygous or ARF-nullizygous Eμ-Myc mice also overexpressed Mdm2. This suggested that more than one member of the ARF/p53/Mdm2 pathway could be altered in these tumors and that the pathway was not strictly linear but must branch to other targets. Therefore, Myc activation strongly selects for spontaneous inactivation of the ARF/p53/Mdm2 pathway both *in vitro* and *in vivo* canceling its protective checkpoint function and accelerating progression to malignancy.

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Regulation of cell growth and apoptosis by the Myc/Max/Mad network: Role of Mad1 and targetting of Max during Fas-mediated apoptosis

Bernhard Lüscher[1], Stefanie Gehring[1,2], Regine Kraft[3], Anja Krippner-Heidenreich[1,4], Annette R. Menkel[1,5], Jürgen Mertsching[1], Sabine Rottmann[1], Robert V. Talanian[6], and Hubert Thole[7,8]

[1]Institut für Molekularbiologie, and [7]Kinderklinik, Medizinische Hochschule Hannover, Carl-Neuberg Strasse 1, 30623 Hannover, Germany; [3]Max-Delbrück-Centrum, Proteinchemie, 13092 Berlin, Germany; [6]BASF Bioresearch Corporation, Worcester, MA 01605-4314, USA

Present addresses: [2]DLR Projektträger des BMBF, Südstraße 125, 53175 Bonn, Germany; [4]Institut für Zellbiologie und Immunologie, Universität Stuttgart, 70569 Stuttgart, Germany; [5]Meersmannufer 1, 30655 Hannover, Germany; [8]Solvay GmbH, 30173 Hannover, Germany

Max is the central component of the Myc/Max/Mad network of transcription factors that regulates growth, differentiation, and apoptosis. Whereas the Myc and Mad genes and proteins are highly regulated, Max expression is constitutive and no posttranslational regulation is known. We have found that Max is targeted during Fas-induced apoptosis, resulting in Max dephosphorylation and subsequent cleavage by caspases. Max is cleaved at IEVE(10)-S by caspase-5, thus representing not only the first caspase-5 substrate but also the first substrate that possesses a glutamic acid in the P1 position. Caspase-7 hydrolyzes Max at SAFD(135)-G near the C-terminus. Both sites are also used in vivo. Furthermore caspase-5 cleavage is inhibited by protein kinase CK2-mediated phosphorylation of Max at Ser-11. Thus the observed Fas-mediated dephosphorylation of Max is required for caspase-5 cleavage. Furthermore DNA binding activity of Max/Max homodimers is modulated during apoptosis. Together our findings uncover three distinct processes, i.e. dephosphorylation and caspase-5 and caspase-7 cleavage, that target Max during Fas-mediated apoptosis, suggesting regulation of the Myc/Max/Mad network through its central component.

To systematically examine the function of Mad1 in growth control and during apoptosis, we have generated U2OS cell clones that express Mad1 under a tetracyline regulatable promoter (UTA-Mad1). Mad1 was induced rapidely and efficiently, localized to the nucleus, and bound to DNA as a heterodimer with Max. The induction of Mad1 reduced cellular growth and, more profoundly, inhibited the formation of colonies under limiting dilution conditions. Conditioned medium could overcome this inhibitory effect implying that Mad1 function is regulated by extracellular signals. Potential Mad1 target genes and genes of cell cycle regulators were not affected by Mad1. In addition Mad1 interfered with apoptosis induced by several different stimuli. UTA-Mad1 cells were protected against Fas-, TRAIL-, and UV-induced apoptosis upon induction of Mad1. Furthermore microinjection of Mad1-expressing plasmids into fibroblasts inhibited apoptosis induced by the oncoproteins c-Myc and E1A. Thus Mad1 not only interferes with cellular proliferation but also with apoptosis, which defines a novel aspect of Mad1 function.

Converging oncogenes in cancer

Gerard Evan

UCSF Cancer Center, 2340 Sutter Street, San Francisco,
California 94143-0128.

Cancers arise through accumulation of mutations that compromise control of cell proliferation, differentiation, cell adhesion and apoptosis. Deregulation of the c-*myc* proto-oncogene is a ubiquitous neoplastic mutation that disrupts cell growth control and renders cells independent of mitogens for cell cycle progression. However, activation of c-*myc* also sensitises cells to apoptosis. We believe that this innately contradictory action of c-*myc* acts as a restraint to the propagation of potential tumour cells harbouring activated c-Myc. We have therefore addressed the question: how relevant is c-Myc-induced apoptosis in the restraint of neoplasia *in vivo*?

To address this question we have constructed mice harbouring a switchable Myc protein targeted to specific tissues - specifically, pancreatic islets cells and surpabasal keratinocytes. Activation of c-Myc in pancreatic islet ß cells triggers 100% entry of cells into cell cycle but this is accompanied by massive apoptosis which rapidly overwhelms proliferation resulting in islet involution and acutely diabetic mice. Co-expression of Bcl-2 blocks this apoptosis and, together with c-Myc, leads to profound ß cell hyperplasia. Thus, pancreatic islets exemplify a tissue in which survival factors are in limiting supply and in which c-Myc deregulation could not trigger malignancy without the very early suppression of apoptosis. By contrast, activation of c-Myc in suprabasal skin rapidly leads to a complex neoplastic phenotype comprising hyperplasia with no accompanying apoptosis. Lesions progress, becoming dysplastic, exhibit dramatic neo-angiogenesis and eventually form papillomas. However, these papillomas remain benign because supernumerary keratinocytes continue to migrate outwards and are eventually shed in parakeratotic tiers. Inactivation of c-Myc triggers complete regression of papillomas, indicating that c-Myc is required to initiate and maintain the neoplastic phenotype. Whereas no apoptosis is evident in the papillomas *in vivo*, c-Myc efficiently induces apoptosis in the same keratinocytes *in vitro*, indicating that c-Myc induced cell death is suppressed in intact skin by excess survival signals. This, in turn, raises the possibility that Myc-induced papillomas might remain benign because any invading cells die when they migrate into an inappropriate dermal trophic environment that lacks requisite survival factors. Consistent with this notion, we show that loss of p53, a protein required for efficient Myc-induced apoptosis in keratinocytes, converts benign Myc-induced papillomas into aggressively invasive carcinomas.

Session 3: Regulation of Myc Expression
Chair: Chi Van Dang

Growth function and regulation of c-*myc* in B-cells

Marino Schuhmacher, Franz Kohlhuber, Alexander Pajic, Thomas Albert, Julie Wells[1], Michael Hoelzel, Axel Polack, Bettina Kempkes, Georg W. Bornkamm, Helmut Burtscher[2], Michael Jarsch[2], Ulrich Weidle[2], Peggy Farnham[1], Dirk Eick

Institute for Clinical Molecular Biology and Tumor Genetics, GSF, Marchioninistrasse 25, 81377 Munich, Germany, e-mail: eick@gsf.de; [1]University of Wisconsin, Madison, USA; [2]Roche Diagnostic-GmbH, Pharma-Research, Penzberg, Germany

In Burkitt's lymphoma (BL), *myc* is transcriptionally activated by chromosomal translocation. As a model for *myc* activation in BL cells, we have established a human B-cell line, P493-6, in which *myc* is expressed under the control of a tetracycline-regulated promoter. Turning off *myc* expression leads to G0/G1 arrest in the presence of serum. Re-expression of *myc* activates the cell cycle without inducing apoptosis. Myc triggers the expression of cyclin D2, cyclin E and Cdk4, followed by the activation of cyclin E-associated kinase and hyperphosphorylation of Rb. The Cdk inhibitors p16, p21, p27 and p57 are expressed at low or not detectable levels in proliferating cells and are not induced after repression of *myc*. These data suggest that *myc* triggers proliferation of P493-6 cells by promoting the expression of a set of cell cycle activators but not by inactivating cell cycle inhibitors.

Regulation of proliferation involves the coordination of cell growth (accumulation of cell mass) and cell division. Division of P493-6 cells is strictly dependent on the expression of the conditional *myc* and the presence of foetal calf serum (FCS). We could show that cell growth is regulated by Myc without FCS: Myc alone induces an increase in cell size and positively regulates protein synthesis which is supposed to be one of the causes for cell mass increase. Furthermore, Myc stimulates metabolic activities as indicated by the acidification of culture medium and the activity of mitochondrial enzymes. Myc induces growth of P493-6 cells in the presence of roscovitine, which blocks Cdk2 activity and thereby Rb phosphorylation. These results confirm the model that Myc can induce cell growth uncoupled from cell division.

We have used P493-6 cells for a global search of Myc-regulated genes. Applying the Affymetrix oligonucleotide chip technology and Clontech DNA microarrays we identified a large number of Myc target genes. Many of the genes are involved in cell metabolism and support the notion that Myc triggers growth by upregulationof a large number of genes of various gene classes. A list of so far identified genes will be presented in the poster of Schuhmacher et al., on this workshop.

Upregulation of Myc protein levels by growth factors occurs indirectly by upregulation *myc*'s transcription rate. We have have reconstituted some aspects of *myc* regulation and could show that remodeling and acetylation of chromatin are critical steps in activation of *myc* transcription. *myc* chromatin was reconstituted on stably transfected, episomal, Epstein-Barr virus-derived vectors. Episomal *myc* P1 and P2 promoters show only basal activity but can strongly be induced by inhibitors of deacetylases (trichostatin, butyrate). The effect of *myc* promoter mutations on *myc* gene activity, chromatin structure, and E2F binding was studied. The analysis revealed that the ME1a1 binding site between P1 and P2 is

required for the maintainace of an open chromatin configuration of the dual *myc* promoters. Mutation of the ME1a1 site strongly reduces the sensitivity of the *myc* core promoter region to micrococcus nuclease and prevents binding and pausing of pol II at the *myc* P2 promoter. In contrast, the E2F binding site, adjacent to the ME1a1 site, is required for repression of the P2 promoter, most likely by recruitment of histone deacetylase activity. Mutation of the E2F site activates transcription from the *myc* P2 promoter but not did not activate the adjacent P1 promoter. Chromatin precipitation experiments with E2F-specific antibodies indicate binding of E2F-1, E2F-2, and E2F-4 bind to the *myc* promoter *in vivo*. Taken together, the analyses support a model with a functional hierarchy for regulatory elements in the dual *myc* promoter region that allows individual as well as common regulation of the P1 and P2 promoters.

Myc interacts with VCP, an AAA-family ATPase

Konstantinos Alevizopoulos*, Dubravka Donjerkovic, Frances Shanahan, Emma Lees and Bruno Amati

Cell Signaling Department, DNAX Research Institute, 901 California Avenue, Palo Alto CA 94304
Present Address: Apotech SA, CH-1066 Epalinges, Switzerland

Mammalian Valosine Containing Protein (VCP) and its yeast homolog cdc48p are members of the AAA-family of ATPases. VCP and cdc48 have been associated with a variety of cellular activities such as proteasome-mediated proteolysis, fusion of endoplasmic reticulum vesicles, assembly of Golgi cisternae, endocytosis, signal transduction, apoptosis and cell cycle control. We identified VCP as a Myc-interacting protein in a yeast two-hybrid screen and confirmed this interaction in human cells by co-immunoprecipitation. The Myc-VCP interaction requires a region of the Myc transactivation domain (Myc box 2) that is essential for cellular transformation. Like Myc, VCP also interacts with components of the mammalian SWI/SNF chromatin remodeling complex: BAF155, SNF5 and BRG1. These interactions suggest a novel function of the VCP ATPase in transcriptional control, possibly as a Myc co-activator and/or as a regulator of chromatin remodeling. Functional studies are in progress to address the role of VCP in Myc-regulated transcription and/or growth control.

OMOMYC: A NEW TOOL TO BETTER UNDERSTAND MYC FUNCTIONS

Soucek L.., La Rocca A., Tatò F., Jucker R., and Nasi S., Centro Acidi Nucleici CNR, Università La Sapienza, Rome, Italy.E-mail: s.nasi@caspur.it

The *myc* gene encodes a transcription factor that contributes to tumorigenesis through its effects on cell growth, differentiation and apoptosis. It binds to DNA as heterodimer wih its partner Max; dimerization and DNA binding are dictated by the bHLHZip domain.

We have focused on a mutant Myc bHLHZip domain, Omomyc, that interferes with Myc function. Omomyc has altered dimerization specificity, being able to homodimerize and to efficiently form heterodimers with the c-Myc protein. Omomyc sequesters Myc in complexes with low binding affinity for the Myc/Max DNA recognition sequence, hampers Myc/Max regulated transcriptional activation of artificial promoters, and prevents NIH3T3 fibroblasts proliferation[1]. We investigated the possibility of utilizing Omomyc for reverting Myc dependent cell transformation and for studying Myc function. Since sustained Omomyc expression might be detrimental for cell growth, we referred to theER^{TM} inducible protein expression system. We first analyzed the C2C12 myoblast cell line, a model system for cell differentiation studies, which is well characterized in its response to Myc. OmoMyc expressing and empty retroviruses as control were produced and utilized to infect normal and Myc transformed C2C12 myoblasts. The phenotype of untransformed cells was not affected by Omomyc, whereas falloidin immunofluorescence showed that OmoMyc induced cytoskeletal reorganization of Myc transformed cells. Upon lowering the serum concentration to 0.5% (differentiation medium), C2C12MycOmomycER^{TM} cells underwent a dramatic increase of apoptosis following tamoxifen induction; the apoptotic rate increase was Myc dependent, as it did not occur in C2C12OmomycER^{TM}cells. To determine whether Max was required for apoptosis potentiation by OmoMyc, we resorted to the PC12 cell line, which displays only an inactive, truncated form of Max that is unable to dimerize. A stable clone expressing OmomycER^{TM} was isolated. The clone showed no growth abnormality and differentiated in the presence of NGF. A strong increase in apoptosis, as compared to control PC12 cells, occurred upon serum removal in the presence of tamoxifen; apoptosis was still prevented by addition of NGF. These data show that Omomyc enhances apoptosis regardless of Max and argue that such Myc function can be dissociated from transcriptional activation at Myc/Max binding sites, since apoptosis was potentiated while transcriptional activation was inhibited by Omomyc. Further analysis might reveal new features of the multiple capacities of the Myc protein.

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Control of c-Myc function through translational and post-translational mechanisms

Qiurong Xiao[1], Uri Vaknin[1], Gisela Claassen[1], Mark Gregory[1], Kirsteen MacLean[2], John Cleveland[2] and Stephen R. Hann[1]

[1]Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN, 37232-2175; [2]Department of Biochemistry, St. Judes Children's Research Hospital, Memphis, TN, 38105-2794

Many studies have shown that the c-*myc* gene has a critical role in the control of cellular proliferation and apoptosis; however, the molecular mechanism responsible for these biological activities of c-Myc remains unclear. The prevailing hypothesis is that transactivation through specific E box myc sites (EMS) of genes such as ODC, cad, eIF4E and cdc25A is necessary and sufficient for the biological functions of the c-Myc protein. Alternatively, several studies have shown that c-Myc can repress specific promoters, including certain growth arrest genes, cell adhesion genes and differentiation-specific genes. We previously identified and characterized c-Myc translational and post-translational events and are seeking to understand the role of these events in controlling c-Myc function. The upstream non-AUG-initiated c-Myc1 protein and the c-Myc2 protein, initiated from the first AUG, both can transactivate through EMS sequences. They do differ in their ability to transactivate through a non-canonical enhancer sequence. The c-Myc1 protein transactivates through this enhancer, whereas c-Myc2 is inactive (Hann et al., 1994). The downstream-initiated c-Myc, c-MycS, lacks approximately 100 N-terminal amino acids, including the highly conserved Myc Box I (MBI) region. While c-MycS is unable to transactivate through EMS elements or non-canonical elements, it is as efficient as full-length c-Myc in repression (Spotts et al. 1997; Xiao et al., 1998). Despite the inability to transactivate, c-MycS is capable of stimulating proliferation, inducing anchorage-independent growth, inducing apoptosis and rescuing the c-*myc* null phenotype in fibroblasts (Xiao et al., 1998). In addition, we have shown that cells overexpressing c-MycS caused tumorigenesis in nude mice. Recent collaborative studies demonstrated that c-MycS is actually more effective than c-Myc2 in inducing apoptosis in mouse embryo fibroblasts (MEFs), whereas c-Myc1 is ineffectual. Both c-Myc1 and c-Myc2, however, stimulated proliferation, as demonstrated by cell cycle analysis of MEFs showing that c-Myc1 and c-Myc2 reduced the number of cells in G1 and increased the number in S phase. In contrast, c-MycS had the opposite effect and increased the number of cells in G0/G1 and decreased the number in S phase, indicative of increased apoptosis. Thus, differential translation initiation of c-Myc appears to modulate the apoptotic activity of c-Myc. Taken together, our data also provide direct evidence arguing against the paradigm that all of the biological functions of c-Myc are mediated by transcriptional activation of specific target genes through EMS elements. However, repression of target genes remains a viable alternative mechanism.

In our search for genes repressed by c-Myc we have found that $p21^{CIP1}$ is repressed by c-Myc at the transcriptional level and effectively blocks the induction of $p21^{CIP1}$ by TGFb (Claassen and Hann, 2000). Also, this repression is not dependent on histone deacetylation. Using differential display we have also identified a novel gene which is highly

expressed in c-*myc* null fibroblasts and is repressed by c-MycS within 1 hour of activation. In comparison, c-MycS activation leads to a delayed increase (several hours) in *cad* and eIF-4E expression and a delayed decrease in endogenous c-*myc* expression. Since c-MycS cannot directly activate transcription and autosuppression has previously been determined to occur through an indirect mechanism, the delayed changes in gene expression induced by c-MycS activation are likely to also be through an indirect mechanism. However, c-MycS can also induce rapid repression of gene expression, perhaps through a direct mechanism. Therefore, our working hypothesis is that c-Myc functions by direct target gene repression in addition to a more indirect, delayed activation of target genes, perhaps through repression of an inhibitor of transactivation or through a derepression mechanism. Further studies support a corollary to our hypothesis, that the N-terminal domain of c-Myc2, including MBI, is necessary for modulation of c-Myc stability and activity through post-translational mechanisms, such as phosphorylation (Gregory and Hann, 2000; Chang et al., 2000).

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Regulation of c-*myc* expression by internal ribosome entry

A. E. Willis

Department of Biochemistry, University of Leicester, University Rd. Leicester LE1 7RH UK.
Email aew5@le.ac.uk

Control of protein synthesis is a major regulatory point of gene expression. Most of the control of protein synthesis occurs at the initiation phase of this process and this is thought to be the rate limiting step. There are two mechanism of initiation of translation in eukaryotic cells: i) the cap-dependent scanning mechanism which requires binding of a complex of proteins, the eukaryotic initiation factor 4F, to the ^{7} methyl G at the 5' end of the mRNA and recruitment of the 40S ribosome and scanning to the first AUG codon in good context (Pain 1996). This mechanism of translation initiation is used by most eukaryotic mRNAs. ii) An alternative mechanism which is termed internal ribosome entry. This requires the formation of a complex structural element termed an internal ribosome entry segment (IRES) in the 5' untranslated region of the mRNA, which allows recruitment of ribosomes to the first AUG which may be a considerable distance (e.g. more than 600 nts) from the 5' end of the primary mRNA sequence (Jackson et al 1995).

Many genes whose protein products are associated with growth control have an atypical 5' UTR of more than 200 nts and to date IRESs have been identified in many such eukaryotic mRNAs including FGF-2 (Vagner et al 1995), PDGF (Stein et al 1998), VEGF (Bernstein et al 1997), Pim-1 (Johannes et al 1999) and work from my laboratory has identified an IRES in c-*myc* (Stoneley et al 1998). Interestingly three of the IRESs identified thus far are found in 5' UTRs which have alternative translation initiation start codons involving an upstream non-canonical CUG codon i.e. c-*myc*, FGF-2 and Pim-1 (Willis 1999).

We have shown that the c-*myc* IRES is located in a 340 nt region of RNA downstream of the P2 promoter (Stoneley et al 1998). Our data also show that *c-myc* mRNA translation from P2 initiated messages can occur via the conventional cap-dependent scanning mechanism (West et al 1998) and that both mechanisms contribute to *c-myc* protein synthesis (Stoneley et al 2000a).

Since two mechanisms are used to initiate the synthesis of the c-*myc* protein we have been investigating the cellular situations where the IRES is utilised. There are a number of physiological conditions where the cap-dependent scanning mechanism of translation is reduced including i) following heat shock, ii) during cell stress, iii) during apoptosis, iv) during mitosis, v) following DNA damage. We have shown that the c-*myc* IRES is not used during cell stress, mitosis or heat shock, however it appears to be required to maintain expression of the protein during apoptosis (Stoneley et al 2000b) and following DNA damage (our unpublished data).

Finally we have been investigating whether IRESs are present in mRNAs of other members of the Myc family of genes and data from these experiments will also be discussed.

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Session 4: Myc/Mad Targets and Biology I
Chair: Martine F. Roussel

c-Myc regulation of cellular metabolism and proliferation

Chi Van Dang

Normal mammalian cells use oxygen to generate energy, whereas cancer cells rely on glycolysis for energy and are hence, less dependent on oxygen. The molecular basis for enhanced glycolysis associated with cancers has become clearer with the observations that loss of tumor suppressors and activation of oncogenes can directly alter gene expression that affects metabolism. Through representational difference analysis, we identified lactate dehydrogenase A (LDH-A) as a differentially expressed gene in c-Myc transformed Rat1a fibroblasts. We observed that the c-Myc directly regulates LDH-A and induces lactate overproduction. We, therefore, determined whether c-Myc controls other genes regulating glucose metabolism. In Rat1a fibroblasts transformed by the c-*myc* gene and murine livers overexpressing c-Myc via *in vivo* adenovirus-mediated gene transfer, the mRNA levels of the glucose transporter GLUT1, phosphoglucose isomerase, phosphofructokinase (PFK), glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, enolase A and LDH-A were elevated above those in non-transformed Rat1a cells or control LacZ expressing livers. c-Myc appears to direct transactivate genes encoding GLUT1, PFK, and enolase and increases glucose uptake in Rat1 fibroblasts expressing the ligand-inducible Myc-estrogen receptor chimeric protein. These findings were further corroborated by the diminished expression of these genes in c-*myc* deficient fibroblasts. Enolase A and LDH-A were both independently found by cDNA microarray analysis to be differentially expressed in wild-type fibroblasts as compared to c-*myc* deficient fibroblasts. Nuclear run-on studies indicate that GLUT-1 transcriptional rate is elevated by c-Myc, and GLUT1 expression is increased in Burkitt's lymphoma cell lines, which are characterized by chromosomal translocations of c-*myc*. Hence, our findings suggest that in addition to the physiological induction of glycolysis by hypoxia, deregulated expression of the c-Myc oncoprotein induces glycolysis through the activation of several components of the glucose metabolic pathway.

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A G2/M checkpoint links c-Myc-induced cell growth with cell enlargement and differentiation in human keratinocytes.

Alberto Gandarillas, Jean-Eudes Dazard, Derek Davies*, and Jean-Marie Blanchard.

IGM, CNRS, Montpellier, France. *ICRF, London, UK.

Human epidermis is a powerful system to investigate the molecular control of proliferation and differentiation. Keratinocytes cease proliferation as they leave the epidermal basal layer and differentiate. The relationship between cell cycle and differentiation and its control are, however, poorly understood. We have recently reported that continuous activity of c-Myc drives epidermal stem cells into the proliferative compartment and, after a few rounds of cell division, to differentiate terminally. We have studied the mechanisms by which c-Myc elicits such an effect. Human keratinocytes are known to enlarge as they differentiate. We have found that they also endoreplicate: they continue DNA replication in the absence of cell division due to a block in G2/M upon differentiation, and become polyploid. Activation of c-Myc stimulates this phenomenon and as a consequence, cell growth results not in proliferation but in increased cell size. We are exploring the involvement of cell cycle regulators downstream of c-Myc in controlling this process. A concerted particular regulation of cyclins, Rb, and the pathway P53/MDM2/p21 consistently follows normal and c-Myc-promoted differentiation. Furthermore, inhibition of mitosis rapidly induces terminal differentiation. Taken together, our results indicate that a G2/M checkpoint is critical in determining the keratinocyte cell decision, and link c-Myc-induced cell growth with initiation of differentiation.

Genetic dissection of Myc function in *Drosophila*

P.Gallant, L.A.Johnston*, R.N.Eisenman*, D.Prober*, B.A.Edgar*, M.Dubs, V.Link, C.Li, L.Montero

Universität Zürich, Zoologisches Institut, Winterthurerstrasse 190, 8057 Zürich, Switzerland; *Division of Basic Sciences, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave N, Seattle WA98109-1024, USA

The Myc/Max/Mad network of transcription factors is involved in cell proliferation, apoptosis, differentiation, and neoplasia in vertebrates, but the physiological roles of these proteins, and the pathways through which they act are only beginning to be understood. In order to address these issues in a genetically tractable model organism, we are studying the Drosophila homolog of Myc, Dmyc. Overexpression of Myc in flies induces a massive increase in cell size and accelerates passage through G1 phase of the cell cycle. Conversely, hypomorphic *dmyc* mutations show a number of recessive traits (including small body and bristle size, a delay in development, and clonal competition) that are characteristic for mutations defective in growth. These results suggest the control of protein synthesis and cellular growth as a primary function of Myc. Signalling through the insulin-receptor (Inr) has also been shown to control growth in animals, and therefore we are investigating the genetic interactions between the Myc and the Inr pathways.

In addition to promoting growth, ectopic expression of Dmyc in the developing eye also induces apoptosis, resulting in aberrant adult eye morphology. This phenotype has been exploited in a genetic screen for dominant modifiers of the Dmyc-overexpression phenotype. We have generated 3'200 independent (EP) transposon insertion lines and found 54 of them to act as dominant modifiers of the Dmyc-phenotype. Their molecular and genetic characterisation is currently ongoing.

Gene target recognition among members of the Myc-superfamily and implications for oncogenesis

Rónán C. O'Hagan[1,2] Michael Ohh[1,3], Gregory David[1,2], Ignacio Moreno de Alboran[4,5], Paul Meltzer[6], Frederick W. Alt[4,5], William G. Kaelin Jr.[1,3] & Ronald A. DePinho[1,2]

1 Department of Adult Oncology, Dana Farber Cancer Institute, Boston, MA 02115 USA
2 Department of Medicine and Genetics, Harvard Medical School, Boston, MA 02115 USA
3 Howard Hughes Medical Institute, Dana Farber Cancer Institute, Boston, MA 02115 USA
4 Howard Hughes Medical Institute, Children's Hospital, Boston, MA 02115 USA
5 Department of Genetics, Harvard Medical School, Boston, MA 02115 USA
6National Human Genome Research Institute, NIH, Bethesda, MD 20892-4470

Myc and Mad family proteins regulate diverse biological processes through their capacity to influence gene expression directly. We have shown that the basic regions of Myc and Mad family proteins are not functionally equivalent in oncogenesis and that conserved differences between the basic regions of these proteins determine target gene selection. Moreover, genome-wide expression profile analysis suggests that the Myc and Mxi1 basic regions regulate both common and distinct gene targets involved in diverse processes including the cell cycle, apoptosis, cellular metabolism and genomic stability. These data support the view that the opposing biological actions of Myc and Mxi1 extend beyond reciprocal regulation of common gene targets. Identification of differentially regulated gene targets provides a framework for understanding the mechanism through which the Myc superfamily governs the growth, proliferation and survival of normal and neoplastic cells. For example, the *Cul1* gene, encoding a critical component of the ubiquitin-ligase SCF^{Skp2}, was identified as a direct transcriptional target of Myc. This observation led to subsequent functional and biochemical studies of the role of Cul1 in Myc-dependent cell cycle progression, thus providing a direct link between c-Myc transcriptional regulation and ubiquitin-mediated proteolysis.

Myc is an essential negative regulator of platelet derived growth factor beta receptor transcription: enhancer-dependent mechanism of repression

Keiko Funa[3], Sara K. Oster[1,2], Wilson W. Marhin[1,2], John M. Sedivy[4] and Linda Z. Penn[1,2]

Department of Medical Biophysics[1], University of Toronto, Ontario Cancer Institute[2], 610 University Ave., Toronto, Ontario M5G 2M9. Institute of Anatomy and Cell Biology[3], Goteborg University, Box 420, SE-405 30, Gothenburg, Sweden. Department of Molecular biology, Cell Biology and Biochemistry[4], Brown University, Providence, RI 02912

Platelet derived growth factor BB (PDGF BB) is a potent mitogen for fibroblasts as well as many other cell types. Interaction of PDGF BB with the PDGF β receptor (PDGF-βR) activates numerous signaling pathways and leads to a decrease in receptor expression on the cell surface. PDGF-βR downregulation is effected at two levels, the immediate internalization of ligand/receptor complexes and the reduction in *pdgf-βr* mRNA expression. Our studies show the product of the c-*myc* proto-oncogene regulates *pdgf-βr* mRNA suppression. Both constitutive and inducible ectopic Myc protein can suppress *pdgf-βr* mRNA and protein. Suppression of *pdgf-βr* mRNA in response to Myc is specific, as expression of the related receptor, pdgf α receptor is not affected. We further show that Myc suppresses *pdgf-βr* mRNA expression at the transcriptional level by a mechanism which is distinguishable from Myc autosuppression. The mouse *pdgf-βr* promoter contains a CCAAT motif, whose interaction with NF-Y is essential for transcription. Co-expression of Myc represses *pdgf-βr* luciferase reporter activity, and the CCAAT motif is indispensable for this action. Myc binds NF-Y subunits, YB and YC, as shown by immunoprecipitation of co-transfected COS-1 cells. Interaction is thought to be direct, as Myc binds to in vitro-translated GST-NF-YB and GST-NF-YC, but not to GST-NF-YA fusion proteins. Myc does not alter the efficacy of NF-Y complex binding to DNA; however, Myc represses the transactivation activity of NF-YC when fused to the GAL4 DNA binding domain. Analysis of c-Myc-null fibroblasts demonstrates that Myc is required for the repression of *pdgf-βr* mRNA expression in quiescent fibroblasts following mitogen stimulation. In addition, it is evident that the Myc-mediated repression of *pdgf-βr* mRNA level plays an important role in the regulation of basal *pdgf-βr* expression in proliferating cells. Thus, our studies suggest an essential role for Myc in a negative feedback loop regulating the transcription of the *pdgf-βr* by an enhancer-dependent mechanism.

Session 5: Myc/Mad Targets and Biology II
Chair: Martin Eilers

The proto-oncogene *c-myc* regulates body mass by controlling the rate of cellular proliferation

A. Trumpp[1,*], Y. Refaeli[1], G.R. Martin[2] & J.M. Bishop[1]

[1] The G. W. Hooper Foundation; Department of Microbiology; University of California at San Francisco (UCSF), San Francisco, California 94143-0552; USA

[2] Department of Anatomy and Program in Developmental Biology, UCSF, San Francisco, California 94143-0452; USA

[*] Present address: Swiss Institute of Experimental Cancer Research (ISREC); Ch. des Boveresses 155; CH-1066 Epalinges; Switzerland

Overexpression of the proto-oncogene *c-myc* has been implicated in the genesis of diverse human tumors. The mechanism by which *c-myc* might contribute to tumorigenesis is not certain. It was originally thought that the gene exerts direct control over the cell division cycle by means of the transcription factor that it encodes. But recent work has raised the possibility that the mitogenic effects of the gene are instead secondary to its effects on cellular size. We now report that *c-myc* is essential to the proliferation of cells in several tissues of the mouse, without any apparent intervention by cellular size. We generated a series of mutant mice in which the expression of *c-myc* was incrementally reduced to zero. Partial reductions were accompanied by reductions in the size of the mutant mice, hypoplasia of lymphoid organs and fat tissue, and a reduced rate of cellular proliferation in vitro. A complete deficiency of *c-myc* caused embryonic death by E10.5, prevented fibroblasts and activated lymphocytes from responding to serum and cytokines, respectively, but had no effect on the proliferative response of previously activated lymphocytes to antigen. Neither a partial nor complete deficiency of *c-myc* had any discernable effect on cellular size. We suggest that c-Myc exerts a direct effect on the cell cycle, in response to signals that facilitate intercellular communication in metazoan organisms.

Conserved Functions of the Myc and Mad Proteins

Lenora Loo, Cynthia Yost, Brian Iritani, and Robert N. Eisenman

Division of Basic Sciences, Fred Hutchinson Cancer Research Center

Studies employing *Drosophila* have demonstrated that the fly homolog of the Myc proteins (dMyc) functions in cell growth. Several studies have shown that the growth promoting property of Myc extend to mammalian cells. We have also recently identified a homolog of a mad-related gene in *Drosophila* (dmad). The *Drosophila* Mad is a bHLHZ protein which contains a region with homology to the mSin3 interaction domain (SID) of mammalian Mad family proteins. As expected dMad associates with *Drosophila* Sin3 and Max and represses transcription at a synthetic promoter containing CACGTG binding sites. Analysis of dmad RNA and protein expression indicate that dmad is expressed during embryogenesis in a dynamic fashion. In several larval tissues dMad is associated with non-endoreplicating cells while dMyc expression is associated with endoreplicating cells. Particularly noteworthy is strong expression of the dMad protein in the peripheral and central nervous system during a period corresponding to G1 arrest. Clonal overexpression of dmad in wing imaginal disc cells demonstrates significantly smaller clone size than controls. Indeed cells overexpressing mad are smaller, as determined by forward scatter analysis. We have also determined that transgenic expression of Mad in mouse lymphoid cells generates smaller cells which are profoundly impaired in differentiation. We are currently analyzing the results of targeted deletion of multiple mad family genes in mice and are also attempting to generate null mutations in mad in *Drosophila*.

"Conditional targeting involves *c-myc* gene in proliferative and apoptotic pathways in lymphocytes"

I. Moreno de Alboran[1], R. O´Hagan, F. Gatner*, B. Malynn*, L. Davidson*, R. Rickert+, K. Fajewsky&, F. DePinho§, and F.W. Alt*

[1]Dept. Immunology & Oncology (D.I.O.), Centro Nacional de Biotecnologia, UAM, Campus Cantoblanco 28049, Madrid (Spain)
*Childrens Hospital and Center for Blood Research, Howard Hughes Medical Institute. Harvard Medical School. 320 Longwood Av. Boston MA 02115, USA.
+ University of California at San Diego, San Diego, CA USA.
& University of Cologne. Cologne, Germany.
§ Dana Farber Cancer Institute. 320 Longwood Av. Boston MA 02115, USA

Cell culture studies have implicated the *c-myc* proto-oncogene in cell proliferation, differentiation, and apoptosis[Dang, 1999 #326]. However, analysis of *c-myc* function in differentiated cell types *in vivo* has been constrained by the early embryonic death of *c-myc* deficient mice[Davis, 1993 #226]. We report a *cre-loxp*-based strategy[Thomas, 1987 #274][Gu, 1993 #233] to delete the *c-myc* gene specifically in B lineage cells[Rickert, 1997 #229] in mice and in cultured primary mouse embryonic fibroblasts (MEFs). Mature B cells that lack *c-myc* are viable and exhibit profound resistance to multiple apoptotic stimuli. Upon stimulation with anti-CD40 antibody and interleukin-4, *c-myc* -deficient B cells express early activation markers but fail to proliferate and are arrested in the G0/G1 stage of the cell cycle in the absence of cell death. Furthermore, *c-myc* deficient mature B cells are more resistant to gamma irradiation, etoposide and CD95 induced cell death than normal B cells[Hueber, 1997 #29]. Finally, *c-myc* deficient MEFs show markedly reduced proliferation[Genestier, 1999 #267] and impaired recovery from growth arrest after gamma irradiation. Together, these results show that *c-myc* gene is functionally linked to multiple apoptotic pathways in mature B lymphocytes and participates in the recovery from gamma irradiation induced growth arrest in MEFs.

Insights from transgenic mice of the role of c-Myc in the control of glucose homeostasis

Fatima Bosch, Tura Ferre, and Efren Riu

Department of Biochemistry and Molecular Biology. School of Veterinary Medicine. Universitat Autònoma de Barcelona. 08193-Bellaterra. Spain

Glucose is a major energy source for all mammalian cells. The extracellular concentration of glucose in normal animals is maintained within a very narrow range, despite large variations in the rate of utilization of glucose and in the availability of exogenous glucose. This homeostatic control is achieved by the matching of glucose flux into and out of the extracellular space through the tightly coordinated secretion of insulin and glucagon (1,2). Alterations at the level of ß-cells, liver or skeletal muscle may lead to a disruption of glucose homeostasis and to the development diabetes mellitus (1).

Diabetes mellitus is the most common metabolic disease in humans. It includes a variety of syndromes with distinct etiologies that collectively afflict 2 to 7% of world population. Of these, 5 to 10 % belong to the category of insulin-dependent diabetes mellitus (type 1 diabetes), which generally appears before age 40, frequently in adolescence, and results from autoimmune destruction of insulin-producing cells within the pancreas. The type 1 diabetic patient depends dramatically upon the administration of insulin. Far more common (90-95%) is non-insulin-dependent diabetes mellitus (type 2 diabetes) which, at least in its early stages, is characterized not by insulin deficiency but by the failure of the hormone to act efficiently in target tissues such as skeletal muscle, liver and fat. Unlike type 1, type 2 diabetes is often associated with obesity. All forms of diabetes are also characterized by hyperglycemia, the development of diabetes-specific microvascular pathology in the retina and renal glomerulus, and neurological and macrovascular complications. Chronic hyperglycemia has been postulated to be the main factor responsible for the development of diabetes-specific microvascular and macrovascular complications.

One approach that we are developing in our laboratory to counteract diabetic hyperglycemia is centered in engineering tissues to take up glucose. This may be achieved by increasing glucose phosphorylation by key target tissues, like the liver. The liver has a central role in glucose homeostasis. When plasma glucose is high the liver takes it up, and replenishes depleted glycogen stores (3). During starvation, the liver releases glucose to blood from glycogenolysis and gluconeogenesis. Glucose transport and phosphorylation are the first steps in glucose utilization. However, glucose phosphorylation by glucokinase (GK) seems to be key in the regulation of glucose utilization by hepatocytes.

c-*myc* is a member of a gene family encoding nuclear phosphoproteins that act as transcription factors, which recognize an "E-box" motif with the central consensus sequence CACGTG (4-6). This sequence is also contained in the glucose/carbohydrate regulatory elements located in the promoter of genes coding for some of the liver enzymes of glycolysis and lipogenesis (7,8). We have shown that an increase in c-Myc protein in the liver of transgenic animals leads to an induction of hepatic glycolysis, by increasing the expression of genes coding for enzymes that control the glycolytic pathway, and glycogen synthesis in the absence of cell proliferation and transformation (9). These changes of liver glucose

metabolism led to a reduction of blood glucose and insulin concentrations. Thus, c-Myc is involved in the control of liver carbohydrate metabolism *in vivo*. After an intraperitoneal glucose tolerance test, transgenic mice showed lower levels of blood glucose than controls, indicating that the overexpression of c-Myc led to an increase in blood glucose disposal by the liver. Furthermore, the overexpression of c-Myc counteracted diabetic alterations through its ability to induce the expression of GK and thus hepatic glucose uptake and utilization, and to block the activation of gluconeogenesis and ketogenesis (10). We also studied the ability of c-Myc in counteracting diabetic alterations long-term after destruction of ß-cells in the absence of insulin treatment. Fifty percent of transgenic mice were still alive and looked healthy four months after Stz-treatment while all of Stz-treated control mice died. Insulin was neither detected in serum nor in the pancreatic islets of the Stz-treated mice. Furthermore, Stz-treated transgenic mice showed normalization of serum glucose, triglyceride, ketone body and protein levels. In contrast to Stz-treated control mice, that showed low glucokinase and pyruvate kinase activities, high levels of both enzyme activities were noted in the liver of Stz-treated transgenic mice. In addition, Stz-treated transgenic mice were also able to produce lactate and accumulate glycogen in the liver. In contrast to surviving Stz-treated control mice, which showed about 27% loss of body weight, the surviving Stz-treated transgenic mice only had a reduction of about 6%. These results indicate that c-Myc may counteract diabetic alterations for a long period after destruction of insulin-producing cells. Thus, this study suggest that engineering the liver to increase glucose uptake by overexpressing transcription factors of c-*myc* family might be useful to counteract diabetic hyperglycemia. Furthermore, we have examined whether the hepatic expression of c-*myc* was able to counteract insulin resistance after feeding a high fat diet. In contrast to control mice, which showed an increase of about 40% in body weight, transgenic mice only showed an increase of about 20%, after 3 months on a high fat diet. Control mice were hyperglycemic, hyperinsulinemic, and showed altered glucose tolerance test, indicating that they had developed insulin resistance, whereas normal levels of blood glucose and insulin were observed in transgenic mice. These results indicated that overexpression of c-*myc* in the liver was able to counteract an insulin resistance state. All these results suggest that hepatic overexpression of c-Myc might be useful to counteract the development of type 2 diabetes and obesity.

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Repression of transcription of the p27Kip1 cyclin-dependent kinase inhibitor gene by c-Myc

W. Yang,1 J. Shen,1 Stefania Pianetti,1 M. FitzGerald,1 M. Arsura,1 M. Wu,1 Z. Suldan,2 R. Romieu-Mourez,1 D. W. Kim,1 H. Lee,1 L. P. Freedman,2 and G. E. Sonenshein1

Department of Biochemistry, Boston University Medical School, Boston MA 021181, and Cell Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY 100212

Upon engagement of the B Cell Receptor (BCR) of WEHI 231 immature B cells, an initial increase in expression of c-Myc is followed by a drop, which induces growth arrest and apoptosis. Here we have examined the potential roles of c-Myc in activation and repression. No commensurate activation was noted either in binding to an E-box in EMSA or in RNA expression of the E-box containing genes ODC, alpha-prothymosin or CAD. Upon BCR engagement, expression of the cyclin-dependent kinase inhibitor p27Kip1 protein varied inversely with the changes in c-Myc. The induction of p27 levels has been implicated in both growth arrest and apoptosis of WEHI 231 cells. Here we report that the p27 Kip1 gene is a target of transcriptional repression by c-Myc. The increase in p27 protein was due to increased levels of p27 mRNA and rate of gene transcription. Activity of the TATA-less, Inr-containing p27 promoter could be repressed upon co-expression of c-Myc in WEHI 231 and other cells. Deletion of Myc homology box II in c-Myc ablated repression, while c-Myc "super-repressor", with mutation of Phe115 to Leu, showed enhanced repression. The sequences mediating transcriptional activity and c-Myc repression were mapped. Binding of Max was shown to facilitate c-Myc binding and repression. Previously, c-Myc was shown to sequester p27 protein. Here, we add another layer of complexity in identifying, for the first time, the p27 gene as a target of repression by c-Myc. Lastly, these studies define a new pathway whereby c-Myc mediates control of proliferation and survival of cells through repression of the p27 gene; this pathway likely also plays a role in the reduced p27 expression observed in cancer cells.

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POSTERS

Overexpression of c-Myc in the basal layer of the epidermis induces hyperproliferative lesions with sebaceous differentiation

I. Arnold and F.M. Watt

Keratinocyte laboratory, Imperial Cancer Research Fund,
44 Lincoln´s Inn Fields, London WC2A 3PX, UK

The epidermis is the stratified squamous epithelium that forms the outer covering of the skin. Keratinocytes, the major cell type of the epidermis, are organised into distinct cell layers. Epidermal proliferation depends on divisions within a subpopulation of basal cells, the stem cells, which have an unlimited capacity for self-renewal. A subpopulation of the stem cells differentiate to transit amplifying cells that are destined to withdraw from the cell cycle and further differentiate after a few rounds of division. Keratinocytes undergo terminal differentiation as they move through the suprabasal layers to the tissue surface. In addition to the interfollicular epidermis epidermal stem cells also give rise to hair follicles and sebaceous glands. The function of c-Myc in the skin is controversial. One set of data suggest that in keratinocytes, as in other cell types, the function of c-Myc is to promote proliferation, and that c-Myc down-regulation is a prerequisite for the initiation of terminal differentiation. In contrast to this idea, it was shown recently that in keratinocyte cultures c-Myc stimulates terminal differentiation by driving entry of stem cell progeny into the transit amplifying cell compartment (Gandarillas A. and Watt F.M,. Genes Dev. 11, 2869-2882, 1997).

To address this controversy we are studying the *in vivo* effects of overexpressing c-Myc in the basal layer of the epidermis. We have generated transgenic mice expressing steroid-activatable c-MycER^{TM} (MycER) fusion proteins under the control of the keratin 14 promoter. MycER consists of the ligand binding domain of a mutant oestrogen receptor fused to the C-terminus of c-Myc. MycER lacks intrinsic transactivation activity and responds only to the synthetic steroid 4-hydroxytamoxifen (OHT), but not to oestrogen (Pelengaris S. *et al.*, Molecular Cell 3, 565-577, 1999). In the transgenic mice c-Myc function was induced by topical application of OHT. High copy number mice developed a hyperproliferative skin phenotype with sebaceous differentiation. In advanced stages angiogenesis and apoptosis were observed. A detailed morphological and molecular characterisation of the phenotype will be presented.

"Myc promotes cell size increase in developing chicken limb".

Delgado, M.D[1]., Piedra, E[2], León, J[1] Ros, M[2].
Departamentos de Biología Molecular[1] y de Anatomía y Biología Celular[2], Facultad de Medicina, Universidad de Cantabria. 39011-Santander. Spain.

c-Myc dimerizes with Max and activates transcription of a set de genes, most of them involved in cell cycle progression and protein synthesis. c-Myc is overexpressed in many human tumors and show proliferation promotion effects in different cultured cell types. c-Myc is also involved in apoptosis control, as demonstrated in many cell culture models and *in vivo* models. Recently, it has been shown that c-Myc is also involved in cell size control in *Drosophila* and mammalian B cells.

During development of chicken limbs, expression de c-*myc* is found particularly in chondrogenic tissues. Our previous results are not consistent with a role of c-*myc* in the physiological or experimentally induced apoptosis in the developing limb (Ros et al. *Int. J. Dev. Biol.* 39, 1021, 1995). In order to investigate the effects of enforced Myc expression in limb development we infected developing embryos in limb buds with retrovirus expressing chicken c-*myc* gene. Expression of the transduced c-*myc* was confirmed by *in situ* mRNA hybridization in whole mounts. The overexpression of functional c-Myc was also assessed by the accumulation of *pitchoune/MrDb* in the infected limbs. The ectopic expression of c-Myc resulted in the appearance of enlarged limbs. This was accompanied by increased size of the c-*myc*-overexpressing cells, as assessed by forward scattering. However, the architecture of most Myc-expressing limbs was normal. We conclude that c-Myc increases cell size in this *in vivo* vertebrate model Therefore, c-Myc plays a role not only at the level of cell division control but also in the control of size of animal cells.

In vivo examination of histone acetylation on the c-Myc target gene *cad* using chromatin immunoprecipitation

Scott Eberhardy and Peggy J. Farnham
McArdle Laboratories for Cancer Research, University of Wisconsin-Madison, Madison, WI

We have previously shown that the growth related transcriptional activity of the *cad* gene is mediated by the c-Myc transcription factor. Our current studies are focused on characterizing the mechanism by which Myc activates the *cad* promoter. Other laboratories have recently shown that Myc is associated with proteins containing histone acetyltransferase (HAT) activity. We have found that treatment of quiescent NIH3T3 cells (which have very low levels of c-Myc protein) with the drug Trichostatin A results in an increase in both histone acetylation at the *cad* promoter and an increase in *cad* transcription. Thus inhibition of deacetylases can mimic the action of Myc at the *cad* promoter. Taken together, these results suggest that Myc may activate its target genes by recruiting HAT activity to a promoter, which would result in increased histone acetylation and loosening of higher order chromatin structure, thereby allowing the transcriptional machinery to assemble on a promoter to initiate transcription. To test this model, our laboratory has used the chromatin immunoprecipitation (ChIP) assay to examine the state of histone acetylation at the *cad* promoter in various stages of the cell cycle. We found that there was no significant increase in histone acetylation at the *cad* promoter at the time corresponding to the increase in *cad* transcription. Instead, acetylation of histone H3 was relatively high at the *cad* promoter at all stages of the cell cycle examined. Using an antibody which recognizes any acetylated lysine, we found a modest increase in nonhistone acetylation at the cad promoter. However, we have examined histone acetylation in differentiating cells and have found that a decrease in histone acetylation does correlate with a decrease in Myc binding to the cad promoter. Thus, our results suggest that the mechanism by which Myc regulates transcription of target genes may involve changes histone acetylation in some, but not all experimental systems.

Does Myc activate transcription by promoting histone acetylation?
A Chromatin Immunoprecipitation study.

Scott Frank and Bruno Amati
DNAX Research Institute, 901 California Avenue, Palo Alto CA 94304 U.S.A.

Myc can activate or repress transcription of distinct sets of target genes. Gene activation is mediated by dimerization with Max and binding to the E-box consensus sequence CACGTG. However, the molecular mechanisms by which DNA-bound Myc/Max dimers activate transcription remain unclear. The recent discovery that Myc interacts with chromatin remodeling complexes such as the histone acetyltransferase GCN5 and the associated cofactor TRRAP suggests that localized changes in chromatin structure may mediate Myc function. To evaluate Myc-dependent changes in histone acetylation we have established a chromatin immunoprecipitation (ChIP) protocol that allows us to evaluate Myc binding and histone acetylation at multiple promoters *in vivo*. We have discovered that Myc binds to promoter regions that are highly enriched for acetylated histones H3 and H4. By examining histone acetylation patterns in cells constitutively expressing Myc and in Myc-deficient cells, we are addressing whether Myc is required for histone acetylation at a given subset of promoters.

Biological function of Myc and Myc-S in human primary fibroblasts and identification of Myc target genes by DNA microarray analysis.

Carla Grandori*, Hilary Coller*, Pablo Tamayo, Todd Golub** and Robert Eisenman*.** Fred Hutchinson Cancer Research Center, Seattle* : Whithead Institute**, Cambridge, USA.

In order to examine the biological consequences of Myc activation in a cell background with no alteration of cell cycle control mechanisms, we have introduced the conditional Myc-ER gene in human primary fibroblasts. In this setting, activation of Myc-ER by 4-hydroxytamoxifen results in stimulation of S-phase entry of density arrested and serum starved cells. Several of the known Myc target genes such as MrDb (now named DDX18) and ODC are induced within few hrs following activation as well as when protein synthesis is inhibited, indicating they represent direct response genes. In parallel, we have also analyzed the Myc protein variant Myc-S, previously shown to promote proliferation and apoptosis of immortalized rodent cell lines. In contrast, our results indicate that Myc-S has undetectable activity as an inducer of S-phase or apoptosis of quiescent primary cells, indicating that there are fundamental differences between primary and immortalized cell lines and their requirement for Myc function. Surprisingly, we found that Myc-S, previously considered solely a repressor of specific reporter genes, is instead both a weak activator and even weaker repressor of endogenous target genes. The implication of these results with respect with Myc domains involved in repression and activation will be discussed.

Finally, in order to monitor global changes in gene expression following Myc activation, we have isolated RNA from quiescent and 4-hydroxytamoxifen stimulated Myc-ER cells at eight hr after induction. Using oligonucleotide microarrays we have monitored changes in expression of ~6416 human genes and expressed sequence tags. The results indicate that 27 genes were consistently induced and 9 were repressed. The identity of the genes revealed that Myc may affect multiple aspects of cell physiology altered in tumor cells: cell growth, cell cycle and cell architecture. The identities of the genes and their possible functions will be presented.

Expression patterns and DNA-binding activities of N-Myc/Max/Mad/Rox in differentiating human neuroblastoma cells

Anna Grynfeld[•], Nikita Popov[§], Håkan Axelson[•], and Marie Henriksson[§]

[•]Department of Laboratory Medicine, Molecular Medicine, Lund University, University Hospital MAS, S-205 02, Malmö,Sweden, [§]Microbiology and Tumorbiology Center, Karolinska Institutet, Stockholm, Sweden

Neuroblastoma is a childhood tumor of the sympathetic nervous system. One of the most important genetic aberrations in neuroblastoma is amplification of the *N-Myc* gene, which is found in 40-50% of the tumors with a poor prognosis. We have investigated the expression patterns and DNA binding activities of the other members the Myc/Max/Mad/Rox network in differentiating human neuroblastoma cells. Northern hybridization and RT-PCR analysis show that expression of the *Mad* genes is regulated differentely in neuroblastoma cell lines during differentiation. In cells with *N-myc* amplification, expression of *Mad1* and *Mad4* increases, while *Mad3* expression remains constant. In cells without *N-myc* amplification expression of *Mad1* decreases, while expression of both *Mad3* and *Mad4* increases moderately during differentiation. Expression of *Rox* changed only slightly during differentiation. Analysis of the DNA-binding activity of E-box binding complexes showed that the N-Myc/Max complexes disappear at the later stages of differentiation, consistent with the expression data. In contrast, the DNA-binding activity of Rox/Max complexes to the canonical E-box site (CACGTG) does not reveal any major difference either in different neuroblastoma cell lines or during differentiation. However, binding of Rox/Max to a non-canonical E-box sequence was enhanced at the later points of differentiation, implying that Rox may undergo regulatory modification leading to altered DNA-binding specificity. Taken together, these data show that the expression of the proteins of the Myc/Max/Mad/Rox network are regulated during neuroblastoma cell differentiation, suggesting that these proteins play a role in this process.

Cooperative anti-proliferative signals required for Mad1-induced cell cycle arrest in monocytic cells

Anne Hultquist [1,2], Siqin[1,2] Wu and Lars-Gunnar Larsson[2] 1) Dept. of Genetics and Pathology, University of Uppsala, S-751 85 Uppsala 2) Dept. of Plant Biology, Genetic Center, Swedish University of Agricultural Sciences, S-750 05 Uppsala, Sweden.

The *mad*- and *myc*-family genes encode transcription factors of the basic region/helix-loop-helix/leucine zipper (bHLHZip) family that act as negative and positive regulators of cell growth, respectively. These factors all utilize the bHLHZip protein Max as a necessary heterodimerization partner to bind DNA recognition sequences in order to regulate transcription of their target genes. The *mad*-family genes are, unlike the *myc* genes, predominantly expressed in non-proliferative, differentiated tissues. The Myc/Max/Mad network has therefore been suggested to work as a switch between growth/growth arrest or growth/ differentiation.
To address the role of Mad1 in hematopoietic growth and differentiation, we utilized U-937 cells, which can be stimulated to differentiate into non-dividing monocytes/macrophages with agents such as the phorbol ester TPA. Induced ectopic expression of Mad1, using the tet-activator or lac-repressor systems, was found to retard cell growth but did not result in G_1 cell cycle arrest or differentiation of U-937 cells. As shown previously, TPA-induced differentiation and cell cycle arrest were inhibited in U-937 cells constitutively expressing v-Myc. Ectopic expression of Mad1 in such cells restored TPA-induced G_1 cell cycle arrest, but did not reestablish the differentiation. Mad1 also sensitized the cells for TGF-β1-induced cell cycle arrest. In summary, our results suggest that Mad1 expression alone is not sufficient to induce cell cycle arrest in these cells but requires cooperative anti-proliferative signals provided by cytokines or phorbol ester.

c-Myc hot spot mutations in lymphomas result in inefficient ubiquitination and decreased proteasome-mediated turnover

Fuad Bahram, Natalie von der Lehr, Cihan Cetinkaya and Lars-Gunnar Larsson

Department of Plant Biology, Uppsala Genetic Center, Swedish University of Agricultural Sciences, Box 7080, 750 07 Uppsala and Department of Genetics and Pathology, University of Uppsala, University Hospital, 751 85 Uppsala, Sweden

The *c-myc* proto-oncogene encodes a short-lived transcription factor that plays an important role in cell cycle regulation, differentiation and apoptosis. *c-myc* is often rearranged in tumors resulting in deregulated expression. In addition, mutations in the coding region of *c-myc* are frequently found in human lymphomas, a hot spot being the Thr58 phosphorylation site, a mutation shown to enhance the transforming capacity of c-Myc. It is, however, still unclear in what way this mutation affects c-Myc activity. Our results show that proteasome-mediated turnover of c-Myc is substantially impaired in Burkitt's lymphoma cells with mutated Thr58 or other mutations that abolish Thr58 phosphorylation, whereas endogenous or ectopically expressed wild type c-Myc proteins turn over at normal rates in these cells. Myc Thr58 mutants expressed ectopically in other cell types also exhibit reduced proteasome-mediated degradation, which correlates with a substantial decrease in their ubiquitination. These results suggest that ubiquitin/proteasome-mediated degradation of c-Myc is triggered by Thr58 phosphorylation revealing a new important level of control of c-Myc activity. Mutation of Thr58 in lymphoma thus escapes this regulation resulting in accumulation of c-Myc protein, likely as part of the tumor progression. Mechanisms by which Thr58 phosphorylation triggers c-Myc degradation will be discussed.

Reference:
Bahram et al. Blood 95, 2104-2110, 2000.

YAF2 interacts with and enhances biological activities of the MycN protein

B. Mädge[1], D. Bannasch[1], C. Geisen[2], M. Schwab[1]
[1]DKFZ Department of Cytogenetics, Heidelberg, [2]Institute for Cell biology, Essen

Introduction:

Neuroblastoma is the most common solid childhood cancer with approximately 1 case/100,000 children unter the age of 15. As a genetic marker the *MYCN* protooncogene is amplified in about 1/3 of tumors, and amplified *MYCN* is correlated with poor prognosis.
The MycN protein is a transcription factor that has oncogenic potential (Schwab et al., 1985), stimulates cell growth (Schweigerer et al., 1990) activates target gene transcription (Lutz et al., 1996) and sensitizes neuroblastoma cells for apoptosis (Lutz et al., 1998).
In a yeast-two-hybrid survey we have identified a new MycN interaction partner, Yaf2, (Bannasch, Mädge et al., submitted) which has previously been described as a YY1 interacting protein (Kalenik et al., 1997). Yaf2 binds MycN both *in vivo* in yeast and in *in vitro*.

Results and conclusion:
Transgenic neuroblastoma cells expressing both inducible MycN (Lutz et al., 1996) and constitutive Yaf2 were generated. Several known Myc target gene promoters linked to reporter genes were transiently transfected into these cells. Reporter gene transcription was stimulated following MycN expression. PrT and ODC promoter activity was further enhanced when Yaf2 was expressed. Endogenous ODC and PrT levels were slightly enhanced following cooperative expression of MycN and Yaf2.
Cells expressing MycN have an accelerated cell division rate, and this effect was futher enhanced by Yaf2. Further, the ability of anchorage independent growth of MycN expressing cells was supported by Yaf2. In cells experssing Yaf2 alone neither transcription activation nor growth promotion was detected.
Our data suggest that MycN and Yaf2 cooperate to enhance at least two biological activities of the MycN oncoprotein: Target gene transcription and cellular growth control.

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Myc promotes cell cycle progression via Cul1-dependent degradation of p27

O'Hagan, R.C., Ohh, M., David, G., Moreno de Alboran, I., Alt, F.W., Kaelin Jr., W.G. and DePinho, R.A.

The c-Myc oncoprotein plays an important role in the growth and proliferation of normal and neoplastic cells. The actions of Myc are likely to be executed on many levels given its capacity to regulate many functionally diverse genes governing cell growth, proliferation, survival and differentiation. Genetic and biochemical evidence locates Myc function upstream of or at the Rb-dependent G1/S transition. However, our understanding of the mechanisms through which c-Myc engages the core components of the cell cycle machinery remain incomplete. Using genome expression profile analysis of c-Myc activation we recently identified Cul-1, a critical component of the p27Kip1 ubiquitin ligase complex SCFSkp2, as a direct transcriptional target of c-Myc. Here we demonstrate that one mechanism whereby Myc promotes proliferation is by directly stimulating expression of Cul1 and thereby promoting ubiquitin-dependent degradation of p27. Overexpression of Cul1 or antisense inhibition of p27 markedly alleviates the slow growth phenotype associated with mouse embryo fibroblasts in which c-myc has been deleted by Cre-mediated recombination. Furthermore, Cul1 expression is sufficient to mediate Myc-dependent ubiquitination and subsequent degradation of p27. These genetic and biochemical data implicate Myc in regulation of ubiquitin-mediated proteolysis and suggest that the effects of Myc on cellular proliferation are mediated in part, via Cul1-dependent ubiquitination and subsequent degradation of the CDK inhibitor p27Kip1.

NMR structural characterization of the tumor suppressor Bin1

Antonio Pineda-Lucena & Cheryl H. Arrowsmith

Department of Medical Biophysics, Ontario Cancer Institute, Princess Margaret Hospital, 610 University Avenue, Toronto, ON, M5G 2M9, Canada

The c-Myc protein, which belongs to the Myc family of nuclear oncoproteins, is involved in a large fraction of human malignancies. The most paradoxical revelation from work on c-Myc has been the finding that under certain circumstances the protein is also able to induce programmed cell death. Strikingly, the protein domain involved in both activities seems to map around the same part of the protein, the so-called N-terminal domain. The N-terminal domain (NTD) contains the transcription activation domain (TAD) and regions required for transcriptional repression, cell-cycle regulation, transformation and apoptosis. Until now, problematic biochemical aspects of c-Myc had impeded the identification and analysis of proteins interacting with the NTD, which is difficult to express and isolate in its native form using recombinant biochemical techniques. With the recent identification of several of these NTD-binding proteins, we now have the opportunity to better define the constituents of c-Myc protein complexes and their role in modulating c-Myc activity. In this context, one of the most interesting systems to analyze corresponds to the interaction between the c-Myc NTD and Bin1, which seems to play a remarkable role in the induction of apoptosis. Bin1 is a phosphoprotein with features of a tumor suppressor that is ubiquitously expressed normally but missing or altered in the majority of tumor cells examined to date. In particular, exon 12A sequences abolish the ability of Bin1 to inhibit malignant transformation by c-Myc. Similarly, these sequences abolish the ability to induce programmed cell death in melanoma cells that endogenously express exon 12A. Here, we present our initial nuclear magnetic resonance data on the structural characterization of two spliced forms of Bin1, with and without the exon 12A sequences. Using this approach, we expect to understand the role of the exon 12A sequences in the interaction between the NTD of c-Myc and the tumor suppressor Bin1.

Mlx, a new Max-like bHLHZip family member: the center stage of a novel transcription factors regulatory pathway?

Alexandre Reymond

Telethon Institute of Genetics and Medicine (TIGEM), San Raffaele Biomedical Science Park, Milan, Italy

The Myc proto-oncogene family members have been identified as the cellular homologs of the transforming oncogene of avian retroviruses. They encode central regulators of mammalian cell proliferation and apoptosis, and they associate with the bHLHZip protein Max to bind specific DNA sequences and regulate the expression of genes important for cell cycle progression. The other family members Mad1, Mxi1, Mad3, Mad4, Mga and Rox (Mnt) antagonize their activities. The Mads and Rox compete with Myc in heterodimerizing with Max and in binding to the same specific target sequences. These Mads:Max and Rox:Max dimers repress transcription through binding to the mSIN3 corepressor protein and by tethering histone deacetylase-containing complexes to the DNA.

In a screen for Rox interactors we isolated Mlx, a new Max-like bHLHZip family member. Mlx is able to homodimerize and heterodimerize with Mad1, Mad4, Rox and two new bHLHZip proteins, Mio and Mir. Mlx is therefore at the center of a network of newly identified E-box binding complexes. Moreover these heterodimers function as transcriptional repressors or transcriptionals activators showing that this network, like the Max network, will have positive and negative constituents. Interestingly the newly identified bHLHZip proteins, Mio and Mir, display a similar restricted pattern of expression during organogenesis suggesting a possible role of these proteins in development. We are currently studying the function of this emerging transcription factors regulatory pathway in cell growth and differentiation.

Increased insulin sensitivity and prevention of obesity by hepatic overexpression of c-Myc in transgenic mice

Efren Riu

The transcription factor c-*myc* is involved in the control of liver carbohydrate metabolism *in vivo*, by inducing hepatic glucose uptake and utilization, and blocking the activation of gluconeogenesis, in the absence of cell proliferation and transformation. These changes in liver glucose metabolism led to a reduction of blood glucose and insulin concentrations. Thus, transgenic mice overexpressing c-Myc in the liver mimicked a situation similar to that observed in high insulin sensitivity states. In the present study, we examined whether the hepatic expression of c-*myc* was able to counteract insulin resistance after feeding a high fat diet. In contrast to control mice, which showed an increase of about 40% in body weight, transgenic mice only showed an increase of about 20%, after 3 months on a high fat diet. Furthermore, control mice were hyperglycemic and hyperinsulinemic, indicating that they had developed insulin resistance, whereas normal levels of blood glucose and insulin were observed in transgenic mice. In addition, when an intraperitoneal glucose tolerance test was performed, transgenic mice fed a high fat diet showed lower levels of blood glucose than controls. These results were consistent with normalization of hepatic GK and L-PK enzyme activities, that led to a normalization of hepatic glucose-6-P, lactate and glycogen content in these transgenic mice. Furthermore, transgenic mice presented a marked reduction in serum levels of triglycerides and free fatty acids, which were similar to those observed in control mice fed a standard diet. These results indicated that overexpression of c-*myc* in the liver was able to counteract an insulin resistance state. Thus, all these results suggest that hepatic overexpression of c-Myc might be useful to counteract the development of type 2 diabetes and obesity.

$p27^{KIP1}$ EXPRESSION IN AGGRESSIVE B-CELL MALIGNANCIES IS A WARNING SIGNAL OF MALFUNCTION OF THE p16 AND p53 TUMOR SUPPRESSOR PATHWAYS.

Sanchez-Beato M, Saez AI, Navas IC, Algara P, Mateo M, Villuendas R, Camacho F, Sánchez E, Piris MA

Molecular Pathology Program, Carlos III National Centre for Oncological Research. Madrid, Spain. Dept. of Genetics and Pathology, Virgen de la Salud Hospital, Toledo, Spain.

p27 over-expression in a group of aggressive B-cell lymphomas with a high proliferative index and adverse outcome has been demonstrated by previous studies. This anomalous expression of p27 was associated with the absence of p27/CDK2 and formation of p27/Cyclin D3 complexes where p27 has been demonstrated to be inactivated. In this study we have linked the molecular study of the p53 and p16 genes with the immunohistochemical analysis of p27 expression, in a group of aggressive B-cell lymphomas, to analyze the relationship between p53 and p16 silencing and p27 anomalous over-expression. Twelve cases with p27 high expression were identified in a series of 46 patients. In all these cases (except one), inactivation of p53/ARF, p16/Rb or both pathways was found. More frequently, inactivation of the p16 gene was identified (8/12). Analysis of the clinical relevance of the simultaneous inactivation of the p16/Rb, p53/ARF pathways and p27 protein was also performed. The group with simultaneous p53 and p16 inactivation together with p27 abnormal over-expression showed an increase in proliferative activity (p: 0.006).

In conclusion, these data show that p27 anomalous expression is associated with inactivation of p16/Rb or/and p53/ARF pathways in aggressive B-cell lymphomas. This finding is consistent with a scenario of CKIs competing for CDK4 binding, where the absence of p16 or p21 allows the redistribution of p27 from complexes containing CDK2, where p27 is active, to others containing Cyclin D3, where p27 is inactive, stabilized, and detectable by immunohistochemical techniques. However, not all cases with p53 or p16 silencing show p27 accumulation, which indicates the existence of additional factors in explaining this finding. One candidate for this role is *c-myc*, which have been shown to contribute to cell cycle progression by inducing p27 sequestration mediated by cyclin D1 and D2 induction and leading to p27 stabilization. Indeed, c-myc over-expression is a regular finding in Burkitt's lymphomas, and may be observed in some large B-cell lymphomas. The presence of c-myc or other possible targets is currently being investigated.

Inactivation of *Pten* and *Cd95* (*Fas*) is crucial to drive *c-Myc* activation towards the development of γ-radiation-induced mouse thymic lymphomas

Javier Santos, Michel Herranz, Mónica Fernández & José Fernández-Piqueras

Departamento de Biología. Laboratorio de Genética Molecular Humana. Facultad de Ciencias. Universidad Autónoma de Madrid. 28049-Madrid. Spain.

Key words: *c-Myc*, *Pten*, *Cd95*, γ-irradiation, mouse thymic lymphomas.

Abstract

Loss of heterozygosity (LOH) analysis performed in 68 γ-radiation-induced primary thymic lymphomas of F1 hybrid mice, provided evidence of significant LOH on chromosome 19 in a region that contains the *Cd95* and *Pten* genes. Here, we demonstrate that both genes are inactivated in 13 of 31 (41.9%) early lymphomas and, more frequently, in frank lymphomas (58/68, 85.3%, for *Cd95*; 54/68, 79.4%, for *Pten*). Interestingly, we also found *c-Myc* over-expression in a significant fraction of early (22/31, 71%) and frank (56/68, 82.3%) thymic lymphomas. These data suggest that *c-Myc* up-regulation constitutes an essential oncogenic stimulus in early stages, and that subsequent inactivation of *Cd95* and *Pten* is required for γ-radiation-induced thymic lymphoma progression.

MYC Target Genes in Cell Cycle Regulation and Cell Growth Control

Marino Schuhmacher, Franz Kohlhuber, Michael Hölzl, Alexander Pajic Martin S. Staege, Georg W. Bornkamm, Axel Polack & Dirk Eick
GSF-Research Centre, Institute of Clinical Molecular Biology, Munich, Germany

To investigate the role of c-*myc* in cell growth and cell cycle control we established a human B-cell line (P493-6) with a conditional, tetracycline-regulated c-*myc*. P493-cells proliferate dependent on the conditional c-*myc* and arrest in G1 after repression of c-*myc*.

Myc activation in the presence of foetal calf serum (FCS) leads to induction of cyclinD, cdk4, cyclinE, accompanied by cdk2 activation and Rb-phosphorylation. In this situation the cells enter the cell cycle and start to proliferate. In the absence of FCS, however, cell division is blocked and Rb-phosphorylation can no longer be detected.

Although Myc cannot induce proliferation in the absence of FCS in P493-6-cells, it is sufficient to stimulate cell growth: Myc induces an increase in cell size and protein synthesis. Furthermore, Myc stimulates metabolism, as indicated by the expression of LDH-A and the activity of mitochondrial enzymes. This was confirmed by blocking the activity of cdk2 with roscovitine and still observed an increase in cell size. Thus, control of cell growth is a function of Myc that is uncoupled from cell cycle entry. The Myc protein appears to be a key player in the processes how cell growth and cell division are coordinated and the cell line P493-6 is a valuable tool to study this.

The cell line P493-6 is also an ideal system to search for new Myc regulated genes. By combining nuclear run-on techniques with Microarray filters, we could identify new potential Myc targets. As TGF-β inhibits proliferation of P493-6 cells in the presence of high Myc levels, we have also performed Microarray experiments with TGF-β treated cells. A comparism of the expression patterns reveals a set of genes that are common targets of Myc and TGF-β, but which are regulated in the opposite direction. One example is *GADD45*, a gene which is involved in regulation of cell cycle arrest. Transcription of *GADD45* is repressed by Myc, but induced after addition of TGF-β. Comparing these expression patterns may help to understand which genes are important for cell cycle control and contribute to tumour formation by c-MYC.

LIST OF INVITED SPEAKERS

Bruno Amati	Cell Signaling Department. DNAX Research Institute. 901 California Avenue, Palo Alto, CA.94304 (USA). Tel.: 1 650 858 7528. Fax: 1 650 496 1200. E-mail: bruno.amati@ dnax.org
Donald E. Ayer	Huntsman Cancer Institute, University of Utah. 2000 Circle of Hope, Salt Lake City, UT.84112-5550 (USA). Tel.: 1 801 581 5597. Fax: 1 801 585 1980. E-mail: don.ayer@hci. utah.edu
Fátima Bosch	Dept. of Biochemistry and Mol. Biology. School of Veterinary Medicine, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona (Spain). Tel.: 34 93 581 10 43. Fax: 34 93 581 20 06. E-mail: fatima.bosch@ uab.es
Michael Cole	Dept. of Molecular Biology. Princeton University, Princeton, NJ.08544 (USA). Tel.: 1 609 258 59 36. Fax: 1 609 258 4574. E-mail: mcole@molbio.princeton.edu
Chi Van Dang	Department of Medicine. The Johns Hopkins University School of Medicine. 720 Rutland Avenue, Baltimore, MD.21205 (USA). Tel.: 1 410 955 2773. Fax: 1 410 955 0185. E-mail: cvdang@jhmi.edu
Dirk Eick	Institute for Clinical Molecular Biology and Tumor Genetics, GSF. Marchioninistrasse 25, 81377 Munich (Germany). Tel.: 49 89 7099-512. Fax: 49 89 7099 500. E-mail: eick@gsf.de
Martin Eilers	Institute of Molecular Biology and Tumour Research, Philipps-Universität Marburg. Emil Mannkopff Str2, 35033 Marburg (Germany). Tel.: 49 6421 286 410. Fax: 49 6421 285 196. E-mail: eilers@imt.uni-marburg.de
Robert N. Eisenman	Division of Basic Sciences. Fred Hutchinson Cancer Research Center. 1100 Fairview Avenue North (mailstop A2-025), P.O. Box 19024, Seattle, WA.98109-1024 (USA). Tel.: 1 206 667 4445. Fax: 1 206 667 6522. E-mail: eisenman@fred.fhcrc.org
Gerard Evan	UCSF Cancer Center. 2340 Sutter Street, San Francisco, CA.94143-0128 (USA). Tel.: 1 415 514 0438. Fax: 1 415 514 6779. E-mail: GEvan@cc.ucsf.edu
Peggy Farnham	University of Wisconsin Medical School. 1400 University Ave, Madison, WI.53706 (USA). Tel.: 1 608 262 2071. Fax: 1 608 262 2824. E-mail: farnham@oncology.wisc.edu

Peter Gallant	Universität Zürich, Zoologisches Institut. Winterthurerstr. 190, 8057 Zürich (Switzerland). Tel.: 41 1 635 48 12. Fax: 41 1 635 68 20. E-mail: gallant@ zool.unizh.ch
Stephen R. Hann	Dept. of Cell Biology. Vanderbilt University School of Medicine, Nashville, TN.37232-2175 (USA). Tel.: 1 615 343 4344. Fax: 1 615 343 5791. E-mail: steve.hann@ mcmail.vanderbilt.edu
Javier León	Dpto. de Biología Molecular. Facultad de Medicina, Universidad de Cantabria. Av. Cardenal Herrera Oria s/n, 39011 Santander (Spain). Tel.: 34 942 20 19 52. Fax: 34 942 20 19 45. E-mail: leonj@medi.unican.es
Bernhard Lüscher	Institut für Molekularbiologie, Medizinische Hochschule Hannover. Carl-Neuberg Strasse 1, 30623 Hannover (Germany). Tel.: 49 511 532 4585. Fax: 49 511 532 4283. E-mail: luscher.bernhard@mh-hannover.de
Germana Meroni	Telethon Institute of Genetics and Medicine. Via Olgettina, 58, 20132 Milan (Italy). Tel.: 39 02 21 56 02 33. Fax: 39 02 21 56 02 20. E-mail: meroni@tigem.it
Martine F. Roussel	Department of Tumor Cell Biology. St. Jude Children's Research Hospital. 332 North Lauderdale, Memphis, TN.38105 (USA). Tel.: 1 901 495 3481. Fax: 1 901 495 2381. E-mail: martine.roussel@stjude.org
Gail E. Sonenshein	Department of Biochemistry. Boston University Medical School. 80 East Concord Street, Boston, MA.021181 (USA). Fax: 1 617 638 5339. E-mail: gsonensh@bu.edu
Andreas Trumpp	Swiss Institute of Experimental Cancer Research (ISREC). Ch. des Boveresses 155, 1066 Epalinges (Switzerland). Tel.: 41 21 692 5817. Fax: 41 21 652 6933. E-mail: Andreas. Trumpp@isrec.unil.ch
Anne E. Willis	Department of Biochemistry. University of Leicester. University Rd., LE1 7RH Leicester (UK). Tel.: 44 116 252 33 63. Fax: 44 116 252 33 69. E-mail: aew5@le.ac.uk

LIST OF PARTICIPANTS

Isabel Arnold	Keratinocyte Laboratory. Imperial Cancer Research Fund. 44 Lincoln's Inn Fields, WC2A 3PX London (UK). Tel.: 44 207 269 3527. Fax: 44 207 269 34 17. E-mail: i.arnold@icrf.icnet.uk
Eva Ceballos	Fac. de Medicina. Dpto. de Biología Molecular. Univ. de Cantabria. Cardenal Herrera Oria s/n, 39011 Santander (Spain). Tel.: 34 942 20 19 55. Fax: 34 942 20 19 45
María Dolores Delgado	Departamento de Biología Molecular. Fac. de Medicina. Univ. de Cantabria. Avda. Cardenal Herrera Oria s/n, 39011 Santander (Spain). Tel.: 34 942 20 19 55. Fax: 34 942 20 19 45. E-mail: delgadmd@medi.unican.es
Scott Eberhardy	McArdle Laboratories for Cancer Research, Univ. of Wisconsin-Madison. 1400 University Avenue, Madison, WI.53706 (USA). Tel.: 1 608 262 4844. Fax: 1 608 262 2824. E-mail: seberhar@students.wisc.edu
Ignacio Flores	Cancer Research Institute. 2340 Sutter Street, Box 0128, San Francisco, CA.94143 (USA). Tel.: 1 415 514 0760. Fax: 1 415 502 6779. E-mail: iflores@cc.ucsf.edu
Scott Frank	DNAX Research Institute. 901 California Avenue, Palo Alto, CA.94304 (USA). Tel.: 1 650 858 7503. Fax: 1 650 496 1200. E-mail: scott.frank@dnax.org
Alberto Gandarillas	IGM. CNRS. 1919 Route de Mende, 34293 Montpellier, Cedex 5 (France). Tel.: 33 467 613651. Fax: 33 467 040231. E-mail: agandari@igm.cnrs-mop.fr
Carla Grandori	Fred Hutchinson Cancer Research Center. 1100 Fairview Ave N, Seattle, WA.98109 (USA). Tel.: 1 206 667 2924. Fax: 1 206 667 6522. E-mail: cgrandor@fhcrc.org
Marie Henriksson	Microbiology and Tumorbiology Center. Karolinska Institutet, 17177 Stockholm (Sweden). Tel.: 46 8 728 6205. Fax: 46 8 33 04 98. E-mail: Marie.Henriksson@mtc.ki.se
Anne Hultquist	Dept. of Genetics and Pathology. University of Uppsala, 751 85 Uppsala (Sweden). Tel.: 46 18 661254. Fax: 46 18 558931. E-mail: Anne.Hultquist@genpat.uu.se
Lars-Gunnar Larsson	Dept. of Plant Biology. Uppsala Genetic Center, Swedish University for Agricultural Sciences, Box 7080, 750 07 Uppsala (Sweden). Tel.: 46 18 673342. Fax: 46 18 673389. E-mail: Lars-Gunnar.Larsson@vbiol.slu.se

Britta Mädge	Dept. of Cytogenetics. Deutsches Krebsforschungszentrum. Im Neuenheimer Feld 280, 69120 Heidelberg (Germany). Fax: 49 6221 42 3277. E-mail: b.maedge@dkfz-heidelberg.de
Jorge Martín-Pérez	Instituto de Investigaciones Biomédicas, CSIC. Arturo Duperier 4, 28029 Madrid (Spain). Tel.: 34 91 585 4603. Fax: 34 91 585 45 87. E-mail: jmartin@iib.uam.es
Ignacio Moreno de Alborán	Dept. Immunology & Oncology (D.I.O.). Centro Nacional de Biotecnología, U.A.M.. Campus de Cantoblanco. 28049 Madrid (Spain). Tel.: 34 91 585 45 00. Fax: 34 91 585 06
Satish K. Nair	Rockefeller University and Howard Hughes Medical Institute. Laboratories of Molecular Biophysics, Box 78, New York, NY.10021 (USA). Tel.: 1 212 327 8339. Fax: 1 212 327 8618. E-mail: nairs@rockvax.rockefeller.edu
Sergio Nasi	Centro Acidi Nucleici CNR, Università La Sapienza. P.le A. Moro 5, 00185 Rome (Italy). Tel.: 39 064 991 22 41. Fax: 39 064 991 25 00. E-mail: s.nasi@caspur.it
Rónán C. O´Hagan	Dept. of Adult Oncology. Dana Farber Cancer Institute, Harvard Medical School. 44 Binney Street, Boston, MA.02115 (USA). Tel.: 1 617 632 6097. Fax: 1 617 632 6069. E-mail: ronan_ohagan@dfci.harvard.edu
Linda Z. Penn	Department of Medical Biophysics. University of Toronto, Ontario Cancer Institute. 610 University Avenue, Room 9-625, M5G 2M9 Toronto, ON. (Canada). Tel.: 1 416 946 2276. Fax: 1 416 946 2065. E-mail: lpenn@oci.utoronto.ca
Ignacio Pérez-Roger	Instituto de Biomedicina de Valencia (CSIC). Jaime Roig 11, 46010 Valencia (Spain). Tel.: 34 96 339 1750. Fax: 34 96 369 0800. E-mail: iperez @ibv.csic.es
Antonio Pineda-Lucena	Dept. of Medical Biophysics. Ontario Cancer Institute, Princess Margaret Hospital. 610 University Avenue, M5G 2M9 Toronto, ON. (Canada). Tel.: 416 946 4501. Fax: 416 946 6529. E-mail: antpl@oci.utoronto.ca
Amaya R. Puig	Centro de Investigaciones Biológicas, CSIC. Velázquez 144, 28006 Madrid (Spain). Tel.: 34 91 561 18 00. Fax: 34 91 562 75 18
Bernd Pulverer	Nature. Porters South, 4 Crinan Street, N1 9XW London (UK). Tel.: 44 171 843 4559. Fax: 44 171 843 4596. E-mail: B.Pulverer@nature.com
Janet Reyes	Facultad de Ciencias Biológicas. Universidad Autónoma de Madrid. Cantoblanco, 28049 Madrid (Spain). Tel.: 34 91 397 8203. Fax: 34 91 397 8202. E-mail: janet.reyes@uam.es

Alexandre Reymond	Telethon Institute of Genetics and Medicine (TIGEM), San Raffaele Biomedical Science Park. Via Olgettina 58, 20132 Milan (Italy). Tel.: 39 02 215 60 221. Fax: 39 02 215 60 220. E-mail: reymond@tigem.it
Efrén Riu	Autonomous University of Barcelona, Bellaterra, Barcelona (Spain). Tel.: 34 93 581 16 49. Fax: 34 93 581 20 06. E-mail: ivvba@ blues.uab.es
Margarita Sánchez-Beato	Molecular Pathology Program, Carlos III National Centre for Oncological Research. Ctra. Majadahonda-Pozuelo Km. 2, 28220 Majadahonda, Madrid (Spain). Tel.: 34 91 509 70 54. Fax: 34 91 509 70 55. E-mail: msbeato@cnio.es
Javier Santos	Departamento de Biología. Laboratorio de Genética Molecular Humana. Facultad de Ciencias. Universidad Autónoma de Madrid. Cantoblanco, 28049 Madrid (Spain). Tel.: 34 91 397 50 00. Fax: 34 91 397 82 02
Marino Schuhmacher	GSF-Research Centre, Institute of Clinical Molecular Biology. Marchioninistr. 25, 81377 Munich (Germany). Tel.: 49 89 7099 508. Fax: 49 89 7099 500. E-mail: Schuhmacher@gsf.de
Laura Soucek	Centro Acidi Nucleici CNR, Università La Sapienza. P.le A. Moro 5, 00185 Rome (Italy). Tel.: 39 064 991 22 27. Fax: 39 064 991 25 00. E-mail: laura@allnet.it

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