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CELL BIOLOGY

Distinct Regions of the IL-7 Receptor Regulate Different Bcl-2 Family Members

Jiang Q, Li WQ, Hofmeister RR, Young HA, Hodge DR, Keller JR, Khaled AR, and Durum SK. Distinct regions of the interleukin-7 receptor regulate different Bcl2 family members. *Mol Cell Biol* 24: 6501–13, 2004.

Interleukin 7 (IL-7) is a cytokine produced by the non-lymphoid cells in lymphoid tissues. T lymphocytes require IL-7 as they develop in the thymus. After leaving the thymus, they require IL-7 in peripheral sites. This IL-7 requirement is very stringent, such that congenital defects in components of the IL-7 receptor complex are the most frequent cause of severe combined immunodeficiency.

Our lab and others have shown that a major function of IL-7 is to keep the T cell alive. We also showed that this "trophic" function is largely based on the balance of Bcl-2 family proteins that control mitochondrial integrity. Thus, IL-7 induced the synthesis of Bcl-2 and Mcl-1, which protect mitochondria.

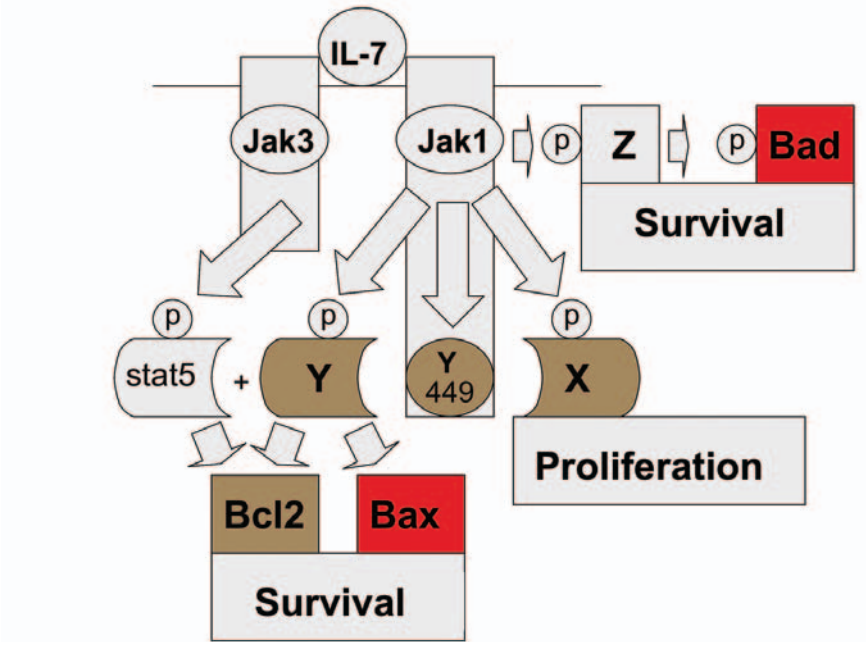

Figure 1. Model showing the interleukin 7 (IL-7) receptor and the survival and proliferation pathways that emanate from it. The major pathway requires Y449 on the intracellular domain of the IL-7 receptor-α (IL-7Rα) chain. Y449 is phosphorylated by Janus kinases Jak1 and Jak3 and then presumably serves as a docking site for unknown adaptors, termed "X" and "Y." These initiate signaling, together with stat5, to the transcription of *bcl-2*, the constraint of Bax to the cytosol, and cell proliferation. A minor signaling pathway is independent of Y449 and, through an unknown kinase "Z," leads to phosphorylation and sequestration of Bad.

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Withdrawal of IL-7 interrupted their synthesis but also posttranslationally activated the death proteins Bax and Bad, which attack mitochondria.

What is the IL-7 receptor signaling pathway leading to these mitochondrial events? The IL-7 receptor complex has been known for some time to include the IL-7 receptor-α (IL-7Rα) chain, which binds IL-7 avidly, and the γ_c chain, which is recruited to this complex. These two receptor chains each bear kinases on their intracellular domains, Jak1 and Jak3, and when these kinases are brought together, they mutually phosphorylate one another and greatly increase their enzymatic activity. However, we do not know their relevant substrates. We know the initial signaling complex. We also know the signaling pathway ends with survival proteins, but we do not understand the middle part.

Whatever signaling pathways emanate from the IL-7 receptor, they are surely important pathways in lymphocytes, and they may well go awry in lymphoma. Several signaling pathways have previously been shown to be activated by IL-7, but none have been shown to be essential. For example, stat5 is activated by IL-7, but its deletion in mice did not have the phenotype of IL-7 insufficiency, which would be manifested as a block in thymic development. The PI3K-AKT pathway and src family kinases are also activated by IL-7, but again, knockout mice have not validated their critical roles. The intracellular domain of IL-7Rα presumably acts as a docking site for the first proteins in the signaling cascade. We hoped that by first identifying the essential intracellular domains of the IL-7Rα chain, we could then find the proteins that interact with these domains and, from there, construct the signaling pathway. Our article (Jiang Q et al. *Mol Cell Biol* 24: 6501–13, 2004) describes our effort to find these critical regions of the IL-7Rα chain.

We first studied IL-7 signals in an IL-7–dependent thymocyte that we had generated from a $p53^{-/-}$ mouse and later verified the principles in T-cell development *in vivo*. Chimeric receptors were produced using the intracellular domain of mouse IL-7Rα coupled to the extracellular domain of human IL-4Rα. This chimeric receptor conferred a vigorous cellular response to human IL-4 that mimicked the response to IL-7, including regulating Bcl-2, Bax, and Bad. The intracellular domain was then mutated to determine the critical signaling regions, which we narrowed down to two very small sites, Box 1 and Y449 (Figure 1—Jak1 is bound to Box 1).

As shown in the figure, we propose that Jak1 initiates two distinct pathways. The major pathway begins with phosphorylation of Y449, which probably serves as a docking site for proteins (yet to be identified) that lead to survival and proliferation. Stat5 docks to Y449 and is phosphorylated and released, then translocates to the nucleus and induces genes. Stat5 augments signaling by increasing Bcl-2 synthesis; however, stat5 is neither necessary nor sufficient. A minor signaling pathway does not require Y449, but leads from Jak1 to inactivation of Bad, and also to the activation of stat1 and stat3.

To evaluate Box 1 and Y449 in T-cell development, we introduced mutated receptors into IL-7Rα$^{-/-}$ hematopoietic stem cells, transferred these stem cells into mice, and observed the effect on thymic development. This confirmed that the major signaling pathway requires Box 1 and Y449, and that a minor pathway is independent of Y449.

We are now seeking to identify the hypothetical proteins (X and Y) that bind Y449, and we are looking for the missing kinase (Z) that phosphorylates Bad at S112 (as described in Li et al. *J Biol Chem* 279: 29160–6, 2004), that is, "the end of the beginning" and "the beginning of the end" (Winston Churchill).

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New Required Intramural Acknowledgement in Publications Will Aid in Tracking of Intramural Contributions to Science

As of July 23, 2005, the NIH Office of Intramural Research requires that all intramural scientists include in the acknowledgements section of all of their publications the following statement:

"This research was supported [in part] by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research."

The wording should be precisely as stated since it will be used to track the publications. Any divergence from this wording will hinder the tracking and lead to possible exclusion of the publication. The [in part] should be removed when the research was fully funded by intramural research.

NIH intramural research makes a large contribution to the world of science. However, that contribution is not always acknowledged or even known, whereas extramural contributions are, and have always been, carefully tracked.

Therefore, the reasons for this new requirement are two fold: First, it will allow for tracking which publications come from intramural scientists despite variation in the way journals report addresses of co-authors. Second, this acknowledgement will highlight the important role that the intramural program plays in a great variety of innovative and collaborative research. As you know, scientists supported with extramural funds already are required to acknowledge NIH in their publications. Any questions should be directed to Tracy Thompson (thompstr@mail.nih.gov).

Human T-Cell Leukemia/Lymphoma Virus Type 1: Playing Hide and Seek

Nicot C, Dundr M, Johnson JM, Fullen JR, Alonzo N, Fukumoto R, Princler GL, Derse D, Misteli T, and Franchini G. HTLV-1–encoded p30^{II} is a post-transcriptional negative regulator of viral replication. *Nat Med* 10: 197–201, 2004.

Humans have specialized immunologic machinery, such as cytolytic T cells, cytokines, chemokines, and antibodies, to fight intracellular and extracellular pathogens. Many pathogens, however, have sophisticated mechanisms to escape human immune surveillance.

The human T-cell leukemia/lymphoma virus type 1 (HTLV-1) (Figure 1, part A) is transmitted sexually or through breastfeeding and causes adult T-cell leukemia/lymphoma (ATLL) or a progressive demyelinating disease called tropical spastic paraparesis/HTLV-1–associated myelopathy (TSP/HAM).

The HTLV-1 genome is composed of two copies of a single-stranded RNA virus whose genome is copied into a double-stranded DNA form that integrates into the host cell genome, at which point the virus is referred to as a provirus. Molecular epidemiological studies indicate a direct correlation between the proviral DNA level and disease occurrence, and some scientists believe that proviral amplification occurs mainly through cell division.

HTLV-1 infects memory (antigen-experienced) CD4$^+$ T cells that are programmed to quickly enter the cell cycle and undergo cell division upon antigen reencounter. Depending on reexposure to an antigen, the CD4$^+$ T cells could divide at any moment and amplify the provirus. However, the viral transcripts in the infected cells *should be* quickly recognized and the cells eliminated by the host immune system. Yet, most T cells carrying the HTLV-1 provirus do not produce detectable viral transcripts. We hypothesized that HTLV-1 might have evolved one or more mechanisms to control its own expression rather than leaving it to the cell cycle process.

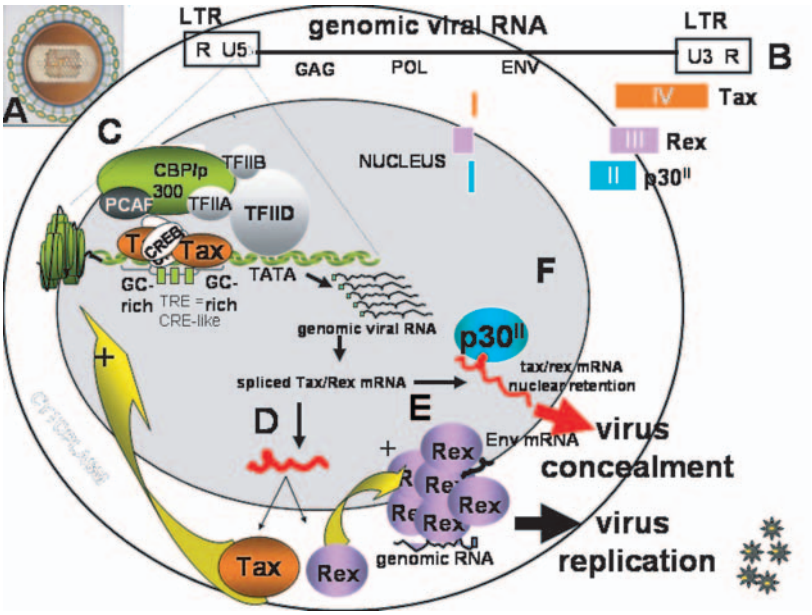

Figure 1. Human T-cell leukemia/lymphoma virus type 1 (HTLV-1) morphology, genomic organization, and replication. *A)* Structure of HTLV-1. *B)* The spliced mRNA encoding the Tax, Rex, and p30^{II} proteins. *C)* A simplified transcription complex on the viral promoter. *D and E)* Tax and Rex production and Rex's effect on the transport of singly spliced and unspliced viral RNAs. *F)* The p30^{II} protein is depicted binding to the Tax/Rex mRNA—it is unknown whether the binding is direct or indirect— and retaining it in the nucleus. LTR, long terminal repeat; CBP/p300, a transcriptional co-activator; PCAF, a transcriptional co-activator; CREB, cAMP response element binding protein; TRE, transcription response element; CRE, cAMP response element; TFIIB, transcription initiation factor IIB; TFIID, transcription initiation factor IID; TFIIA, transcription initiation factor IIA.

HTLV-1 genome expression (Figure 1, part B) begins by transcription from the viral promoter within the long terminal repeat (LTR). The genomic RNA encodes the structural protein Gag and the enzymatic protein Pol, whereas a singly spliced mRNA encodes the Env protein. A unique doubly spliced mRNA encodes two *positive regulators* of viral expression, Tax and Rex (Figure 1, part B). Increasing levels of Tax during infection leads to the recruitment of highly effective transcription complexes to the viral promoter (Figure 1, part C) and full-fledged viral expression. The genomic RNA that encodes the protease, reverse transcriptase, and integrase, as well as the Env protein, is necessary for the production of infectious viral particles. Rex's function is essential in transporting the singly spliced Env and the unspliced genomic mRNAs to the cytoplasm (Figure 1, part C).

We have previously found that HTLV-1 encodes a protein, called p30^{II}, which is generated from a doubly spliced mRNA from the HTLV-1 ORF II proviral sequence (Figure 1, part B) (Koralnik I et al. *Proc Natl Acad Sci U S A* 89: 8813–7, 1992). More recently, we have demonstrated that p30^{II} is a negative regulator of viral expression. The negative effect of p30^{II} on viral replication is not attributable to interference with Tax-mediated transcription from the viral promoter, as overexpression of Tax or Rex cDNA alone is unable to counteract the negative effect of p30^{II} on proviral expression. However, when Tax and Rex are expressed from the unique doubly spliced mRNA derived from the proviral clone, p30^{II} exerts a negative

post-transcriptional effect. Quantitative reverse transcriptase (RT)–PCR analysis of viral messenger mRNA species demonstrated that the cytoplasmic level of the Tax/Rex mRNA is decreased by $p30^{II}$. Importantly, we found that $p30^{II}$ is a nuclear-resident protein unable to shuttle in and out of the nucleus. In addition, we demonstrated that $p30^{II}$ binds to the doubly spliced Tax/Rex mRNA. Therefore, the Tax/Rex mRNA is retained in the nucleus. As expected, overexpression of $p30^{II}$ in HTLV-1–infected T-cell lines also decreases viral replication by decreasing the level of Tax.

Immune T cells continuously patrol lymphoid and nonlymphoid tissues in search of "foreign" signals. We propose that $p30^{II}$ may help HTLV-1 to hide when cells divide. This hypothesis could explain the paradox that most T cells carrying the provirus do not produce detectable viral transcripts. How $p30^{II}$ expression is regulated and whether inhibition of $p30^{II}$ function may reveal hidden infected cells to immune surveillance are unknown. Thus, further investigation on the basic molecular mechanisms of $p30^{II}$ activity might teach us how to reduce or even eradicate virus-infected cells and prevent disease.

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MOLECULAR BIOLOGY

An Unliganded Thyroid Hormone Nuclear β Receptor Induces Pituitary Tumors

Furumoto H, Ying H, Chandramouli GV, Zhao L, Walker RL, Meltzer PS, Willingham MC, and Cheng SY. An unliganded thyroid hormone beta receptor activates the cyclin D1/cyclin-dependent kinase/retinoblastoma/E2F pathway and induces pituitary tumorigenesis. *Mol Cell Biol* 25: 124–5, 2005.

Thyroid stimulating hormone–secreting pituitary tumors (TSH-omas) represent about 2% of all pituitary adenomas in humans. Because diagnosis occurs late in the natural course, curative surgical resection of TSH-omas remains under 50%. Our study focused on understanding the molecular genetic events underlying the pathogenesis of TSH-omas and on identifying potential molecular targets for their early diagnosis.

Clues concerning genetic alterations leading to TSH-omas emerged when dominantly negative mutated thyroid hormone β receptors (TRβ) were identified in several patients with TSH-omas (Ando S et al. *J Clin Endocrinol Metab* 86:5572–6, 2001; Ando S et al. *Mol Endocrinol* 15: 1529–38, 2001). TRβ and TRα are ligand-dependent transcription factors that mediate the biological activities of thyroid hormone (T3) in growth, differentiation, and development. Our creation of a *TRβ* knockin mutant mouse harboring a targeted dominantly negative *PV* mutation in the *TRβ* gene locus has provided an opportunity to address the role of TRβ mutants in the pathogenesis of TSH-omas. The *PV* mutation was identified in a patient with resistance to thyroid hormone. It has a frame-shift mutation in the last carboxyl-terminal 14 amino acids that results in the loss of T3 binding and transcription activities. As $TR\beta^{PV/PV}$ mice age, they spontaneously develop TSH-omas with enlarged and highly vascular pituitaries. Pathohistologic analyses indicate that TSH-containing thyroprival cells (enlarged, growth-stimulated, TSH-producing cells with large nuclei) are in the pituitaries of $TR\beta^{PV/PV}$ mice, beginning at the age of approximately 3 months. As these mice age further (beyond an additional 3 months), adenomas staining positively for TSH and varying in size from microscopic foci (microadenomas) to almost the entire size of the adenohypophysis (with only a thin rim of remaining normal tissue) also become detectable in the pituitaries.

We have found that $TR\beta^{PV/PV}$ mice exhibit severe dysregulation of the hypothalamic-pituitary-thyroid axis, having a 9- to 15-fold increased thyroid hormone level that is associated with a 400- to 500-fold elevated circulating serum TSH level. To address whether persistent elevation of TSH due to the loss of the negative regulation by thyroid hormone underlies the development of TSH-omas, we took advantage of mice devoid of all TRs ($TR\alpha1^{-/-}TR\beta^{-/-}$ mice) that also exhibited severe dysfunction of the pituitary-thyroid axis. In contrast to $TR\beta^{PV/PV}$ mice, the size of the pituitaries in the $TR\alpha1^{-/-}TR\beta^{-/-}$ mice was significantly reduced (20% to 30%), despite a virtually identical loss of the negative regulation of TSH by thyroid hormone in both mutant mice, as compared with the age-matched, wild-type mice. Moreover, histological examination of the pituitary indicated no apparent focal adenomas. These results suggest that the loss of the negative regulation of TSH by thyroid hormone alone is not sufficient to induce TSH-omas.

The distinction between the pituitary growth phenotypes of $TR\beta^{PV/PV}$ and $TR\alpha1^{-/-}TR\beta^{-/-}$ mice provides us with a tool to identify genes that are differentially expressed to promote tumor growth in $TR\beta^{PV/PV}$ mice. Indeed, using cDNA microarrays, we identified *cyclin D1* as one of 12 genes involved in growth and cell proliferation pathways whose

expression is mostly activated in $TR\beta^{PV/PV}$ mice but is repressed or not significantly changed in $TR\alpha1^{-/-}TR\beta^{-/-}$ mice. Additional biochemical and cell-based studies indicated that the increased expression of *cyclin D1* at both the mRNA and protein levels leads to the activation of the cyclin-dependent kinase (CDK)/retinoblastoma (Rb)/E2F pathway that mediates, at least in part, the aberrant proliferation of thyrotrophs in $TR\beta^{PV/PV}$ mice.

How does a TRβ mutant (PV) protein activate the expression of cyclin D1, a critical regulator of the cell cycle and tumorigenesis? A cell-based transcriptional study showed that the liganded wild-type TRβ repressed the *cyclin D1* promoter activity, whereas PV, which does not bind T3, failed to do so, resulting in constitutive activation of the promoter activity. Analysis of the *cyclin D1* promoter revealed the absence of apparent thyroid hormone response elements, but the presence of DNA binding sites for transcription factors AP1, E2F1, SP1, TCF/LEF, and cyclic AMP response element binding protein (CREB). These results suggest that wild-type TRβ and mutant PV could regulate the *cyclin D1* promoter activity via protein-protein interaction with these transcription factors. Indeed, *in vitro* GST pull-down and cell-based co-immunoprecipitation assays demonstrated the physical interaction of TRβ and mutant PV with CREB. Promoter deletion analysis indicates that when the CREB binding site is deleted, the repression by the liganded TRβ or the activation by PV is lost. This suggests that PV, like TRβ, is tethered to the CREB-containing promoter through the physical interaction with CREB on the *cyclin D1* promoter, resulting in constitutive activation of cyclin D1 expression (Figure 1).

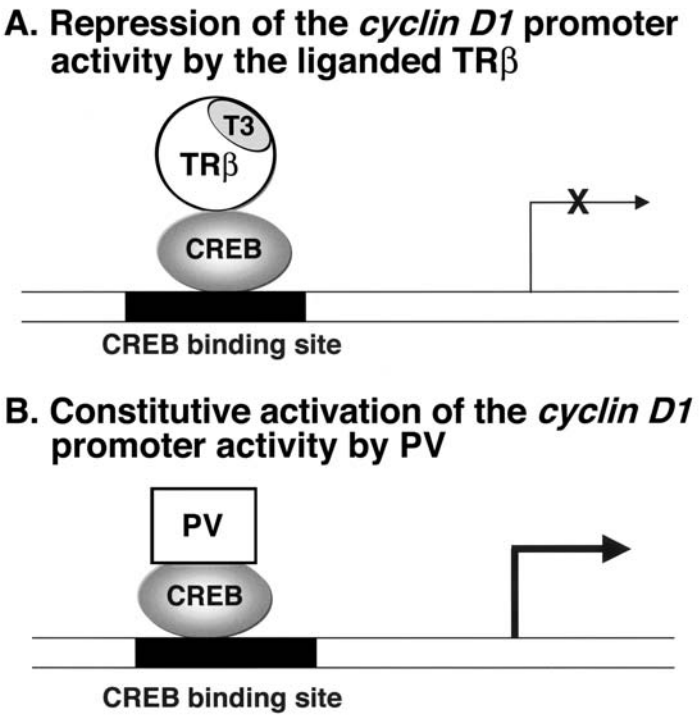

Figure 1. An unliganded TRβ mutant constitutively activates the expression of *cyclin D1* promoter, leading to aberrant growth of thyrotrophs in $TR\beta^{PV/PV}$ mice. *A)* A T3-bound wild-type TRβ acts to repress the expression of the *cyclin D1* promoter via tethering to DNA-bound cyclic AMP response element binding protein (CREB) on the promoter of *cyclin D1*. *B)* PV protein, a TRβ mutant that has lost T3 binding activity, tethering to DNA-bound CREB, cannot function to repress the *cyclin D1* promoter, leading to constitutive activation of the *cyclin D1* promoter. The increased expression of cyclin D1 activates the cyclin-dependent kinase (CDK)/retinoblastoma (Rb)/E2F pathway, resulting in aberrant proliferation of thyrotrophs and tumors in the pituitaries of $TR\beta^{PV/PV}$ mice.

Thus, the *in vivo* evidence suggests that mutation of the $TR\beta$ gene is one of the genetic events mediating the pathogenesis of this disease. Importantly, the present study reveals a novel molecular mechanism by which an unliganded TRβ mutant induces pituitary tumorigenesis *in vivo* and provides mechanistic insights into the pathogenesis of TSH-omas in patients. This study also raises the possibility that the mutated TRβ could serve as a molecular marker for diagnosis.

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Chromatin Function: A Network of Competitive Interactions Between Nucleosome Binding Proteins

Catez F, Yang H, Tracey KJ, Reeves R, Misteli T, and Bustin M. Network of dynamic interactions between histone H1 and high-mobility-group proteins in chromatin. *Mol Cell Biol* 24: 4321–8, 2004.

The ability of regulatory factors to access their binding sites in nucleosomes affects DNA-related processes in chromatin, such as transcription, replication, repair, and genetic recombination. These processes are modulated by nuclear proteins, such as the linker histone H1 and members of the high mobility group (HMG) families, which reduce or enhance the access to nucleosomes, respectively. Cells from higher eukaryotes contain about eight H1 variants in amounts sufficient to bind to over 80% of the nucleosomes. HMGs are nuclear proteins that interact with nucleosomes and change the "architecture" of chromatin. The HMGs are subdivided into three families, HMGA, HMGB, and HMGN, each with a distinct structural motif through which it binds to chromatin. An interplay between H1 and HMGs, which have opposite effects on chromatin compaction, could be part of the mechanism that imparts flexibility to chromatin fiber and could play an important role in determining the cellular phenotype.

Together with Dr. Tom Misteli of the Laboratory of Receptor Biology and Gene Expression, who developed approaches to study the chromatin binding of proteins in living cells, we are investigating whether chromatin binding proteins compete for nucleosome binding sites. In living cells, chromatin binding proteins such as H1 and HMG proteins are in constant motion and interact only transiently with their chromatin binding sites. The proteins bind to chromatin in a "stop-and-go" process, the "stop" being the stage in which they are bound and the "go" being the stage in which the protein moves from one binding site to another. The length of the "stop" and the "go" steps depends on the binding constant of the protein for a particular site. Their sum determines the overall apparent mobility of the protein in the nucleus. For example, a protein that binds strongly to chromatin will display a low mobility, as the binding step will slow down its movement. The mobility of proteins in living cells is experimentally monitored by techniques such as fluorescence recovery after photobleaching (FRAP).

To test for competition between H1 and HMG chromatin binding proteins, we microinjected individual HMG proteins (HMGA, HMGB, or HMGN) into cells expressing fluorescent H1, and compared the mobility of H1 in injected cells versus non-injected cells. We reasoned that if HMG and H1 compete with each other for chromatin sites, an increase in the intracellular concentration of HMG should decrease the binding of H1. Indeed, for all three HMG protein families, the mobility of fluorescent H1 was higher in injected cells than in control cells. Thus, each HMG protein weakens the binding of H1 to chromatin. The inhibition is specific since HMG mutant proteins, which do not bind to chromatin, did not affect the binding of H1 to chromatin. HMG proteins decreased the chromatin binding of H1 in a dose-dependent fashion, a typical feature of molecular competition. These results indicate that HMGs and H1 proteins compete with each other for chromatin binding sites. Because all members of the three HMG families bind to nucleosomes and compete with H1, we next tested whether the HMGs compete with each other. While competitions were observed between HMG proteins of the same family (e.g., between HMGN1 and HMGN2), no competition could be observed between HMGs from different families (i.e., HMGB with HMGN). Thus, there is a certain level of specificity in the competition. Because HMGs do not compete with each other, we thought that they might work together to reduce H1 binding. Thus, we mixed different HMGs together and monitored the impact of their injection, individually or mixed, on H1 binding to chromatin. We found that the effect of HMG mixes on H1 binding is stronger than the sum of the effects of each HMG injected separately. Thus, the HMG proteins compete with H1 synergistically.

Our studies indicate that members of four families of structural proteins that regulate chromatin structure and activity form a network of competitive interactions on nucleosomes. It is likely that such competitive interactions occur between other classes of chromatin binding factors. The realization that chromatin binding proteins function within a network has important ramifications for understanding mechanisms that regulate the cellular response to biological signals. Conceivably, the cell can tailor a specific response by adjusting the equilibrium of the binding and activity of the various members of a protein network. Thus, the dynamic competition between chromatin binding proteins could affect numerous DNA-related processes and perhaps play a role in the epigenetic regulation of gene expression.

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Translational Immunology Related to Cancer: Meeting Highlights

The Center of Excellence in Immunology (CEI) is one of four Centers of Excellence in the NCI Intramural Research Program (IRP). The mission of the CEI is to foster the discovery, development, and delivery of novel immunologic approaches for the prevention and treatment of cancer and cancer-associated viral diseases. To create opportunities for immunologists to exchange information and to facilitate collaborations, the CEI has initiated an annual series of meetings on cancer-related immunology research. The first of these, "Translational Immunology Related to Cancer," was held September 22 and 23, 2005, in the Masur Auditorium in Building 10. Dr. Jeffrey Schlom headed the Scientific Organizing Committee, which included Drs. Steven Rosenberg, Tom Waldmann, Ron Gress, and Jay Berzofsky. Speakers included scientists in the CCR and distinguished invitees from the extramural community. More than 600 scientists attended, and approximately half were from outside the NIH. Highlights of presentations by senior CEI members are described below. A complete list of the presentations at the meeting can be seen at http://web.ncifcrf.gov/events/tirc/ and all the talks can be viewed at http://videocast.nih.gov/PastEvents.asp?c=1.

Figure 1. The poster session, held on the afternoon of September 22nd, drew a large crowd and fostered a lively scientific exchange.

Introductory Comments

Dr. Robert Wiltrout, head of the CEI, introduced the center, describing a faculty of approximately 200 scientists with expertise in the fields of immunology, virology, and epidemiology. Dr. Wiltrout emphasized the importance of this venue for integrating basic and clinical research and accelerating the translation of research into treatments for cancer. Dr. Andrew von Eschenbach next welcomed participants and described his vision of the NCI IRP as an organization that "serves, catalyzes, and leads the scientific field with research and rapid translation."

Monoclonal Antibodies and Cytokine Session

Dr. Tom Waldmann of the Metabolism Branch provided an overview of his pioneering work on interleukin-2 (IL-2) and IL-15. He discussed his work in defining the contrasting role of these cytokines in regulating the immune response, developing humanized antibodies to components of the receptors, arming the antibodies with toxins or isotopes, and using them as treatments for cancer and other diseases of the immune system.

Dr. Wiltrout presented translational studies on the use of IL-2 in combination with either IL-12 or antibodies to CD40. He showed data from phase I studies in which IL-12 and IL-2 were administered to patients with advanced solid tumors. As part of studies directed toward identifying potential mechanisms of cytokine action, his group in the Laboratory of Experimental Immunology investigated the role of interferon-γ (IFNγ) induction in the antitumor effects of various cytokines. He shared intriguing findings that indicate contrasting roles for IFNγ in tumor growth and metastases, as well as antitumor immunity.

Dr. Ira Pastan of the Laboratory of Molecular Biology discussed his work targeting specific antigens on cancer cells, including CD22, mesothelin, and the epithelial growth factor (EGF) receptor, with recombinant immunotoxins (RITs). In phase I trials, treatment with an immunotoxin targeting CD22 has resulted in complete remissions in a high percentage of patients with refractory hairy-cell leukemia. He indicated that phase II trials using RITs to treat hairy-cell leukemia, other B-cell malignancies (BL22), mesothelioma, ovarian cancer (SS1P), and glioblastoma (TP38) are in progress or will open soon. He also showed follow-up work identifying new targets on the surface of malignant cells, as well as approaches to increase the potency and reduce the immunogenicity of RITs.

Cell-Based Therapy Session

This session began with Dr. Nick Restifo of the Surgery Branch discussing innovative, preclinical studies of adoptive cell therapy in murine models of melanoma. Dr. Steven Rosenberg, head of the Surgery Branch, followed, demonstrating how information from these animal studies has been successfully translated into

treatments for patients with advanced melanoma. He showed exciting results of a cell-based therapy of refractory metastatic melanoma: In brief, there has been a 50% response rate using this approach. Dr. Rosenberg also described work in using this approach to treat lung, breast, and prostate cancer.

Vaccine Session

Dr. Jay Berzofsky of the Vaccine Branch talked about improving vaccine design by blocking inhibitory pathways in the immune system. He described elegant basic research demonstrating natural killer T cell (NKT)–dependent increases in secretion of transforming growth factor-β (TGF-β) by myeloid cells that suppress induction of CD8-positive cytotoxic T lymphocytes. He then followed up with a discussion of translational work showing the role of this pathway in tumor immunosurveillance in animal models and the ability to improve cancer vaccine efficacy by blockade of this pathway.

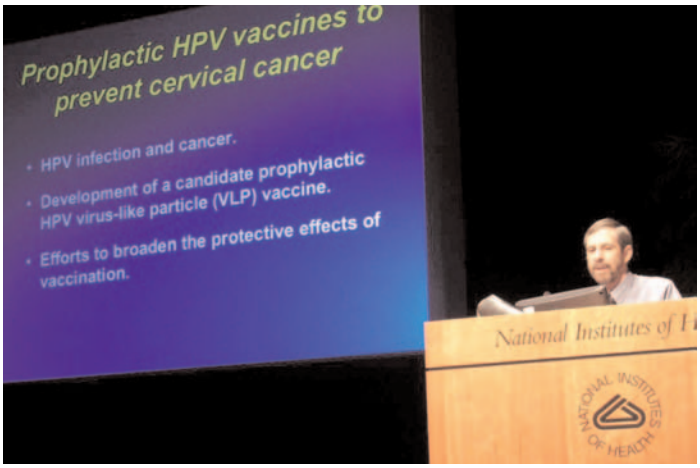

Figure 2. Dr. Doug Lowy presents work on vaccines to prevent infection with human papillomavirus at the Translational Immunology Related to Cancer Meeting.

Dr. Jeffrey Schlom of the Laboratory of Tumor Immunology and Biology spoke about comprehensive studies with vaccines developed in his laboratory. He described bench-to-bedside work with TRICOM vaccines, a class of vaccines designed to enhance immune response through inclusion of genes for three T-cell costimulating molecules and tumor antigens into viral vectors. He showed data illustrating a dramatic increase in avidity of cytotoxic T lymphocytes in response to TRICOM vaccines and also reported exciting new work that combines primary vaccination with intratumoral vaccination, or targeted radiation. Dr. Schlom concluded with the suggestions that sole dependence on reduction in extensive tumor burden in early clinical trials could be inappropriate to monitor vaccine efficacy and that increased survival in patients with limited disease might be a more appropriate paradigm.

Dr. James Yang of the Surgery Branch gave a provocative overview of the challenges in developing cancer vaccines to successfully treat solid tumors. He noted the low percentages of objective responses (3.9%) of patients receiving tumor vaccines in the published literature and described a number of strategies his group is pursuing, aimed at increasing successful outcomes. He also cautioned that while an increase in antigen-specific T cells is an important component of vaccine response, tumors often recur despite this increase. He emphasized that understanding how to induce existing tumor-reactive T cells to reject tumor cells is another important milestone in designing effective vaccines for cancer.

Dr. Doug Lowy of the Laboratory of Cellular Oncology described the bench-to-bedside progression of a vaccine designed to prevent infection with human papillomavirus (HPV). Cervical cancer is the third most common cancer among women worldwide and is caused by infection with a subset of HPVs. Phase II trials with commercially produced versions of this vaccine have demonstrated a high level of protection against HPV infection. Phase III trials, directed by Dr. Allan Hildesheim in the Division of Cancer Epidemiology and Genetics, are under way.

Transplantation and Antitumor Therapies

Dr. Ron Gress of the Experimental Transplantation and Immunology Branch presented studies that provide new insight into mechanisms mediating production and maintenance of T lymphocytes of thymic and peripheral origin. These include advances in understanding regulation by cytokines such as TGF-β, IL-7, IL-15, insulin-like growth factor-1 (IGF-1), and keratinocyte growth factor (KGF). He provided compelling evidence underscoring how this information is contributing to clinical strategies that use immunotherapies such as tumor vaccines and adaptive cell transfer to treat T-cell lymphopenia associated with transplantation, cancer, HIV, and chemotherapy.

Dr. Crystal Mackall of the Pediatric Oncology Branch spoke of her work on homeostasis of T regulatory cells during chemotherapy-induced lymphopenia. She demonstrated that lymphopenic patients can have enrichment, of relative frequency, of CD4-positive, T regulatory cells and showed that IL-2 increased T regulatory cells, particularly during lymphopenia. In contrast, IL-7 increased CD4 and CD8 cells, but did not increase CD4-positive, T regulatory cells. She concluded with the suggestion that depleting T regulatory cells while treating lymphopenia could be a useful tool to amplify antitumor response.

Additional Information and Plans for Future Meetings

Information on the CEI can be found at http://home.ccr.cancer.gov/coe/immunology/. Dr. Diana Linnekin can be contacted at dlinnekin@ncifcrf.gov for additional information on CEI activities.

The next meeting in the CEI series on Cancer and Immunology is tentatively planned for the fall of 2006 and will deal with "Basic Immunology Related to Cancer."

■ **Diana Linnekin, PhD**

In Situ Analyses of Genome Instability in Breast Cancer

Chin K, de Solorzano CO, Knowles D, Jones A, Chou W, Rodriguez EG, Kuo WL, Ljung BM, Chew K, Myambo K, Miranda M, Krig S, Garbe J, Stampfer M, Yaswen P, Gray JW, and Lockett SJ. *In situ* analyses of genome instability in breast cancer. *Nat Genet* 36: 984–8, 2004.

The molecular mechanisms involved in normal cells progressing to invasive metastatic cancer have been well studied in cell culture. These include changes in genome stability and reactivation of telomerase enzyme activity—a critical event that protects the ends of chromosomes, allowing cancer cells to divide indefinitely. Reporting in the September issue of *Nature Genetics*, my colleagues at the University of California, San Francisco, and I provide new insight into how breast cancer develops by examining the way the genome loses its integrity within the cells in the tumor itself. The study traces the stage-specific evolution of genome instability that occurs during the benign-to-malignant transition in breast cancer. Central to the study was the development of computational algorithms to detect intact, individual cell nuclei from 3D images of thick tissue (20 to 40 μm) and to enumerate the copy numbers of fluorescence *in situ* hybridization (FISH)–labeled DNA sequences in each nucleus (Figure 1). Calculation of the copy number variation from cell to cell gave a measure of genome instability in the tissue. We found that genome instability was low in benign and hyperplastic tissues, but dramatically increased in ductal carcinoma and invasive cancers (Figure 2). The highest levels occurred at the same time that cells reactivated telomerase and attained the capacity to continue dividing. This raises a new paradigm of episodic, as opposed to progressive, genome instability in the progression to cancer.

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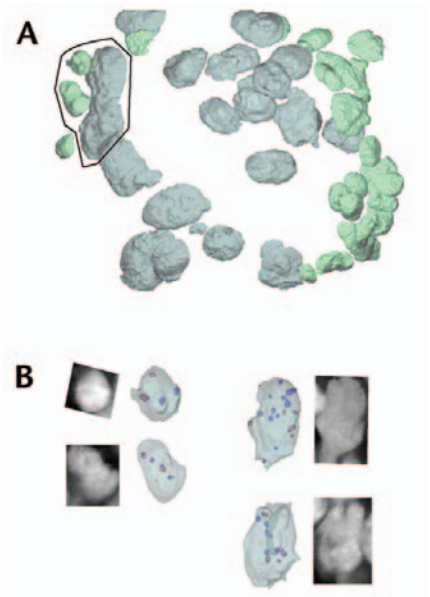

Figure 1. *A)* Surface rendering of the intact cell nuclei in an image showing spatial segregation of near diploid and polyploid cells. The larger nuclei, which had > 2 copies of both a locus in the centromeric region of chromosome 1 and a locus at 20q13, are shown in gray and formed a central cluster surrounded by smaller nuclei with ≤ 2 copies of both loci, in green. *B)* Expansion of the region inscribed in panel A showing the individual nuclei with their surface opacities reduced to reveal the fluorescence *in situ* hybridization (FISH) signals. Cyan dots are the chromosome 1 centromere (1cen) signals, and magenta dots are 20q13 signals. Note that the two green nuclei have two copies of both 1cen and 20q13, whereas the gray nuclei have > 2 copies of both. Raw confocal slices through the nuclei are shown beside each nucleus. Reprinted with permission from Macmillan Publishers Ltd: *Nature Genetics* 36: 984–8, 2004, online supplemental information, http://www.nature.com/ng/journal/v36/n9/suppinfo/ng1409_S1.html, copyright 2004.

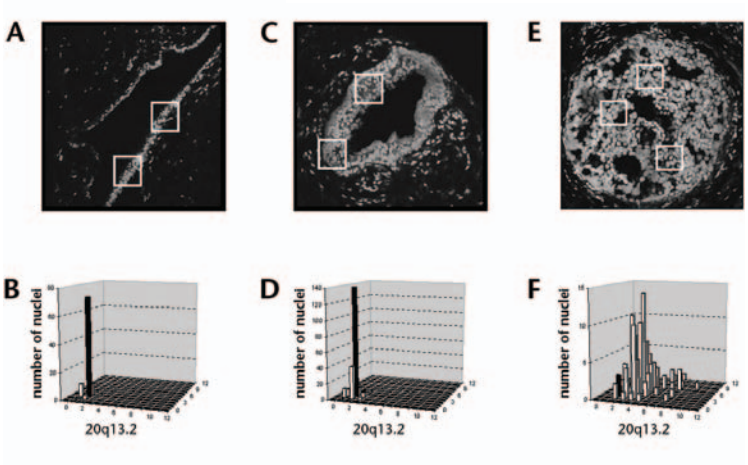

Figure 2. 2D-confocal YO-PRO-1 images and bivariate copy number histograms of chromosome 1 centromere (1cen) and 20q13.2 signals in 3D-images from breast tumors. White bars in each histogram indicate the numbers of cells, with copy numbers specified on the X- and Y-axes. The black bar in each histogram indicates the number of cells with 2 copies each of 1cen and 20q13.2. *A)* A 2D-confocal YO-PRO-1 image of normal ductal epithelium taken midway through a 30 μm thick tissue section. The white squares (100 μm × 100 μm) indicate regions for which 3D-confocal images were acquired for copy number analysis. *B)* A bivariate copy number frequency histogram of the number of copies of 1cen and 20q13.2 in the regions indicated in *(A)*. Over 90% of nuclei show two copies of 1cen and 20q13.2. *C)* A 2D-confocal YO-PRO-1 image of a usual ductal hyperplasia. *D)* A bivariate frequency histogram of 1cen and 20q13.2 copy numbers measured for the regions indicated in *(C)*. While most cells showed two copies of 20q13.2, 22% of cells had only one copy of 1cen. *E)* A 2D-confocal YO-PRO-1 image of a ductal carcinoma *in situ* (DCIS) showing an expanded duct filled with heterogeneous tumor cells. *F)* A bivariate frequency histogram of 1cen and 20q13.2 copy numbers measured for the regions indicated in panel *(E)* showing substantial genomic instability. Reprinted with permission from Macmillan Publishers Ltd: *Nature Genetics* 36: 984–8, 2004, copyright 2004.

Involvement of Chaperones in the Control of DNA Replication of Bacterial Plasmids

Das N and Chattoraj DK. Origin pairing ("handcuffing") and unpairing in the control of P1 plasmid replication. *Mol Microbiol* 54: 836–49, 2004.

Chromosomal DNA replication is a highly controlled process in all growing cells. This is also true for multicopy plasmids of bacteria. If the plasmid copy number is not controlled, plasmid-free cells could result. The risk of generating plasmid-free cells increases with reductions in the copy number, but too high a copy number is also undesirable. Cells with too high a copy number grow more slowly, as plasmids are a metabolic burden to the host. These slow-growing cells can be outnumbered by faster-growing plasmid-free cells. Therefore, for stable maintenance, it is essential that plasmids maintain their copy number within narrow limits. They achieve this with remarkable economy using one or two plasmid-encoded genes.

Extrachromosomal existence, although risky, allows plasmids the opportunity for horizontal transfer to other species and genera. A classic example is the movement of the T_i plasmid from *Agrobacterium* to the nucleus of plant cells where they cause crown gall tumor. It is therefore not surprising that the host factors upon which plasmids depend are often well-conserved and ubiquitous proteins, such as molecular chaperones.

The involvement of molecular chaperones in DNA replication initiation has been known for quite some time from studies on bacteriophages and plasmids. In the best-studied cases, the chaperones remodel the initiators, resulting in activation of the initiator itself (Wickner S et al. *Nature* 350: 165–7, 1991) or release of replication factors trapped in an inactive state (Alfano C and McMacken R. *J Biol Chem* 264: 10699–708, 1989).

Two mechanisms are generally found to control the replication of bacterial plasmids. The first one is by antisense RNA. It was at the NIH that antisense control was discovered by Tomizawa J. and Itoh T. in studies of ColE1 (*Proc Natl Acad Sci U S A* 78: 6096–100, 1981). The second one, which we have studied in plasmid P1, is achieved by DNA repeats (iterons; block arrows, Figure 1) that bind the only plasmid-encoded initiator protein, Rep. The binding of Rep to iterons not only allows replication initiation but is also involved in controlling the frequency of replication initiation in the cell cycle. The control involves increasing the replication frequency in cells with too few plasmids and decreasing it in cells with too many plasmids (Das N et al. *Proc Natl Acad Sci U S A* 102: 2856–61, 2005). It appears that this inverse relationship of replication frequency to copy number (negative feedback control), which is central to maintaining the copy number within narrow limits, can be achieved by simple Rep dimerization and its dissociation with the help of chaperones.

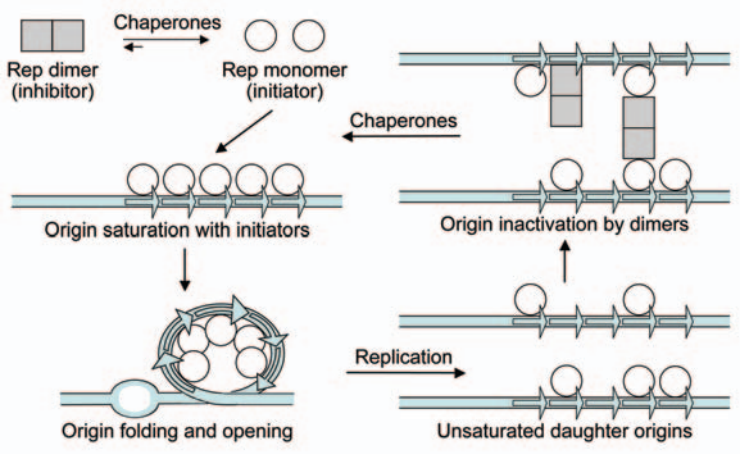

Figure 1. Control of initiation of DNA replication by chaperone-mediated dissociation of initiator dimers. Initiator (Rep) binds to its multiple sites (iterons; block arrows) in the origin as a monomer. Saturation of the binding sites leads to origin folding that absorbs one superhelical turn of DNA and opens the adjacent AT-rich region, which encourages initiation. Upon replication, the initiator monomers are titrated by the daughter origins so that no origin is saturated. Replication also causes an increase of Rep synthesis. The nascent Rep dimerizes readily and competes with monomers for origin binding and/or mediates origin pairing. These events prevent origin folding. Chaperone-mediated dimer dissociation and increase of monomer allow origin saturation and folding, which preclude origin pairing. The replication cycle can thus repeat.

Rep is active as an initiator only as a monomer. However, Rep dimers readily form and require remodeling by chaperones (DnaK, DnaJ, and GrpE) to dissociate and serve as initiators. Our study shows that dimerization not only reduces monomers but could play an active role in control as well.

We believe a primary mechanism for negative control is by pairing of Rep-bound daughter origins (handcuffing). Pairing apparently prevents origin folding, which opens the origin, a prerequisite for replication initiation. Pairing of DNA sites is common in transcriptional repression and activation both in bacteria and eukaryotes. Pairing must be reversed if replication is to resume. In transcriptional control, pairing is reversed simply by adding inducer or depleting the pairing protein. In our case, the chaperones seem to do the trick.

When plasmid copy number increases, the increase in *rep* genes also raises total Rep concentration and thereby encourages more dimer formation. In

fact, the dimer-to-monomer equilibrium ratio increases with increases in total protein. The dimers serve as inhibitors by effecting handcuffing and/or by directly binding to iterons. By converting dimers to monomers, the chaperones help by both reducing handcuffing and aiding origin saturation by increasing monomer concentration.

Replication control must ensure initiation but prevent premature reinitiation. Chaperones, by modulating the dimer-to-monomer (inhibitor-to-initiator) ratio, achieve both these goals. In this scheme, the economy of control does not seem to compromise the efficiency of control.

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■ TRANSLATIONAL RESEARCH

Altered Localization of RXRα Coincides with Loss of Retinoid Responsiveness in Human Breast Cancer

Tanaka T, Dancheck BL, Trifiletti LC, Birnkrant RE, Taylor BJ, Garfield SH, Thorgeirsson U, and De Luca LM. Altered localization of retinoid X receptor alpha coincides with loss of retinoid responsiveness in human breast cancer MDA-MB-231 cells. *Mol Cell Biol* 24: 3972–82, 2004.

Retinoids are natural and synthetic vitamin A derivatives, which regulate development, cell proliferation, and differentiation. Retinoids also act as cancer preventive agents and are presently being used successfully to treat certain types of cancer. Although many studies have shown retinoids to be effective in inhibiting cancer cell growth *in vitro* and *in vivo*, the clinical usage of vitamin A derivatives is currently limited by the requirement of relatively large dosages to reach therapeutic efficacy. It is also likely that the responsiveness of cancer cells to retinoids diminishes in relation to malignant progression. Indeed, the growth inhibitory effects of retinoids have been observed in estrogen receptor–positive breast cancer cells of low malignancy, whereas the effectiveness of retinoids has been observed to diminish in highly malignant breast cancer cells that were estrogen receptor negative. The existing hormonal, chemotherapeutic therapies have provided a substantial improvement for the survival of patients with localized breast cancer; however, treatment for metastatic breast cancer remains palliative. Thus, there is an urgent need to understand the mechanism of retinoid resistance to develop therapeutic agents for metastatic breast cancer.

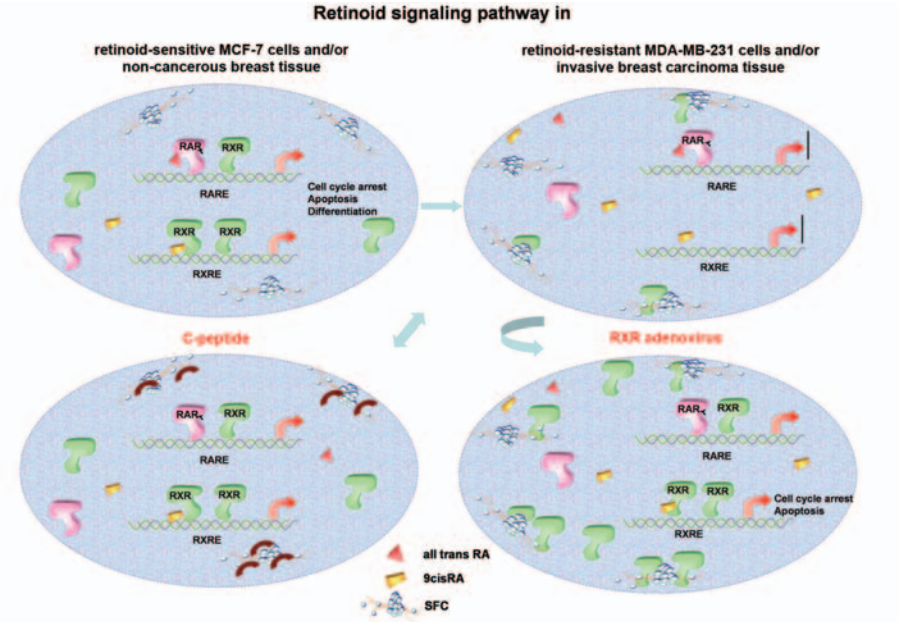

Figure 1. Retinoid X receptor-α (RXRα) intranuclear localization is a critical factor in retinoid responsiveness. RXRα localizes throughout the nucleoplasm in retinoid-sensitive normal cells and MCF-7 cells of low malignancy. In contrast, RXRα is sequestered in the splicing factor compartment (SFC) and silenced in MDA-MB-231 cells; consequently, retinoid signaling is shut off in these cells. To reverse the lack of responsiveness to retinoid, which itself is attributable to the sequestration of RXRα, two separate approaches were taken. RXRα C-terminus–specific peptide to MDA-MB-231 cells facilitated redistribution of RXRα throughout the nucleus, increasing RXR-homodimer–mediated transactivation upon RXR-ligand treatment. Also, nucleoplasmic overexpression of RXRα in MDA-MB-231 cells infected with RXRα adenovirus resulted in apoptosis in accordance with increased p21 and decreased Bcl-2 expression, restoring the retinoid sensitivity. RA, retinoids; RXRE and RARE, response elements.

The physiological actions of retinoids are mediated through two distinct nuclear receptor families, the retinoic acid receptors (RAR α, β, and γ), each of which binds all-*trans*-retinoic acid or 9-*cis*-retinoic acid, and the retinoid X receptors (RXR α, β, and γ), which preferentially bind 9-*cis*-retinoic acid. RARs and RXRs bind to a specific DNA response element (RARE) in the 5′-flanking region of target genes as homodimers or heterodimers, thereby promoting gene transcription.

We studied the loss of retinoid responsiveness from the perspective of subcellular localization of the retinoid receptors. In sharp contrast to RXRα homogeneous nuclear distribution in estrogen receptor–positive HMEC and MCF-7 cancer cells, RXRα localized to the splicing factor compartment (SFC) in estrogen receptor–

negative MDA-MB-231 cancer cells. We also found that RXRα localized to the SFC in the connective tissue of invasive breast carcinoma tissue, but not in the epithelial cells. SFC localization was not detected in connective tissues of normal or benign hyperplasia.

Vitamin D receptor B1 (VDRB1), a heterodimerization partner of RXR, is also found in SFC and is redistributed throughout the nucleoplasm upon exposure to its ligand 1,25-dihydroxyvitamin D3. Unlike the ligand-induced dynamic intranuclear mobility of VDRB1, we found that ligand failed to redistribute RXRα from the SFC to the nucleoplasm in MDA-MB-231 cells. This finding allowed us to hypothesize that RXRα might be sequestered in the SFC, thereby contributing to loss of retinoid responsiveness. We demonstrated that RXRα was not localized to active transcription sites in MDA-MB-231 cells but showed extensive colocalization with nascent transcripts in MCF-7 cells. This result was further confirmed by reporter assays when the RXR-selective ligand promoted RXRE (RXR-homodimer target) transactivation in MCF-7 cells but failed to do so in MDA-MB-231 cells. The absence of ligand-dependent transcriptional activation in MDA-MB-231 was not attributable to the reduced RXRα protein expression level because the RXRα level in retinoid-sensitive HMEC cells was the same as that in MDA-MB-231 cells. Thus, we decided to investigate whether altered localization of RXRα could explain the loss of RXRα activity and retinoid responsiveness in the MDA-MB-231 cell line. When MDA-MB-231 cells were infected with adenoviral RXRα, exogenous RXRα was localized throughout the nucleus in addition to the SFC. Nucleoplasmic overexpression of RXRα induced apoptosis in accordance with p21 upregulation and bcl-2 downregulation in the presence of ligand (Figure 1).

Epitope-tagged and a C-terminus deletion mutant of RXRα failed to localize to the SFC, whereas exogenous full-length RXRα did so heavily, indicating that the RXRα C-terminus might play a critical role in shuttling RXRα to the SFC. Delivering RXRα C-terminus–specific peptide to MDA-MB-231 cells facilitated redistribution of RXRα throughout the nucleus, increasing RXR-homodimer–mediated transactivation upon RXR-ligand treatment and ultimately enabling MDA-MB-231 cells to respond to retinoids.

In conclusion, RXRα was found in the SFC in highly malignant breast cancer MDA-MB-231 cells and invasive carcinoma of human breast tissue. These findings suggest that RXRα appears to change its subcellular localization as cells become increasingly malignant. Our study clarifies one possible pathway that participates in loss of retinoid signaling during breast cancer progression and provides the new concept that loss of RXRα activity due to altered localization leads to the loss of retinoid responsiveness in highly malignant breast tumor cells.

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