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NATIONAL INSTITUTES OF HEALTH
DEPARTMENT OF HEALTH AND HUMAN SERVICES

MOLECULAR BIOLOGY

Non-Native Hydrophobic Interactions in a Hidden Folding Intermediate

Feng H, Takei J, Lipsitz R, Tjandra N, and Bai Y. Specific non-native hydrophobic interactions in a hidden folding intermediate: implications for protein folding. *Biochemistry* 42: 12461–5, 2003.

Protein folding is the final step in the transfer of genetic information from DNA to proteins. It is only after proteins appropriately fold that they have the capability to perform their biological functions. In the 1960s, Anfinsen and colleagues at the NIH demonstrated that proteins could fold spontaneously from the unfolded state to the native state under physiological conditions. It was hypothesized that the native structure is the most stable state. This hypothesis provided a theoretical basis for predicting protein structures from their amino acid sequences and Anfinsen was awarded the Nobel Prize

in 1972. However, the detailed process of protein folding remains elusive. Two recent events have made the study of protein folding one of the most important issues in current biology: 1) More than 20 human diseases, termed amyloid diseases, have been found to be related to protein misfolding and precipitation in cells, including Alzheimer's, type II diabetes, and Creutzfeldt-Jakob disease, a human version of the mad cow disease. In several cases, it was shown that partially unfolded intermediates are the major precursors of the amyloid molecule. These precursors are thought to be responsible for the amyloid diseases. 2) The genome project has accumulated a huge number of protein sequences. However, to understand the functional information encoded in these sequences, the structures of proteins are needed. Thus, it becomes highly desirable to understand the relationship between protein sequences and structures so that the structures of proteins can be predicted from their sequences by computational methods. Toward this goal, a structure genomics program has been initiated at the NIH. Physical studies of protein folding are essential because all computer programs for predicting protein structures rely on the force-field parameters derived from the physical studies of protein folding.

Significant effort has been made in characterizing the structures of partially unfolded intermediates. To date, they have been mainly characterized by using amide hydrogen exchange and mutation studies. The amide hydrogen exchange method can measure the hydrogen bond formation in secondary structures through studying the

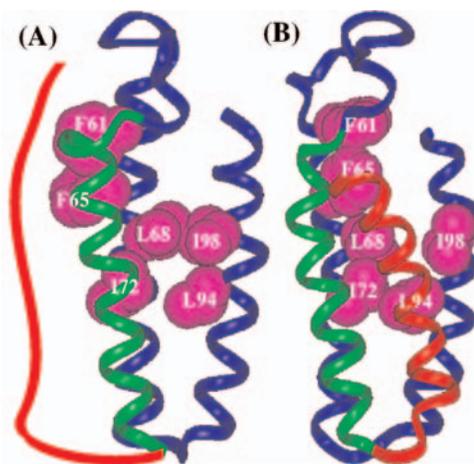


Figure 1. A) Structure of the non-native intermediate of Rd-apocytochrome b_{562} . Represented hydrophobic residues are shown in CPK models. The red coil represents the unfolded N-terminal helix. B) Native structure of Rd-apocytochrome b_{562} .

rates of exchange between amide protons and protons in water. Mutation studies can produce information on side chain interactions by measuring the energetic effect of a mutation on the folding intermediate relative to the native state. These studies, however, can only yield low-resolution structures, and one often assumes that the intermediates have native-like structures in the folded regions.

We identified a partially unfolded intermediate of a four-helix bundle protein, Rd-apocytochrome *b*₅₆₂, a redesigned apoform of an electron transfer protein. The N-terminal helix of this intermediate is unfolded, as determined by the hydrogen exchange method. Given this low-resolution structure, a mutant was designed to substitute the hydrophobic core residues in the N-terminal helix with glycines. These substitutions selectively destabilize the native state, making the intermediate the most stable state without affecting the folded regions.

We have now determined the high-resolution structure of the intermediate

by using multi-dimensional nuclear magnetic resonance (NMR). Although the three folded helices have native-like topology, surprisingly, there are significant non-native hydrophobic interactions among side chains (Figure 1). Nevertheless, we found the non-native intermediate buries approximately $258 \pm 80 \text{ \AA}^2$ more of the solvent-accessible hydrophobic surface than does the putative native-like intermediate with the N-terminal helix unfolded. Our findings provide the first structural evidence in support of the hypothesis that evolution has minimized the exposure of hydrophobic surfaces in folding intermediates by forming non-native hydrophobic interactions to avoid aggregation. The findings also have profound implications for theoretical studies of the folding mechanism of proteins since force-field potentials that allow only native-like interactions have been widely used.

The non-native features of the folding intermediate of Rd-apocytochrome *b*₅₆₂ also have significant implications for understanding the general mechanism of protein folding (Feng H et al. *Proc*

Natl Acad Sci U S A 102: 5026–31, 2005). An important question has been why many folding intermediates populate before rate-limiting transition states. The current molten-globule model suggests that early folding intermediates exist because formation of secondary structures is intrinsically fast while tertiary packing is slow (molten globule model). The Rd-apocytochrome *b*₅₆₂ intermediate now shows that the repair of the *mis*-packed residues cannot be rate limiting because the intermediate was previously shown to form after the rate-limiting step. Instead, we propose that population of early folding intermediates is likely due to the existence of multiple domains that have different folding kinetics. We are currently testing this hypothesis experimentally.

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■ FROM THE DIRECTOR

NCI Director Dr. Andrew von Eschenbach Visits the CCR

In early April, NCI Director, Andrew von Eschenbach, MD, visited Building 41 on the NIH Campus as part of his ongoing efforts to learn more about the science being conducted at the CCR and to discuss its implications and impact in the field of cancer research. CCR scientists are committed to advancing the NCI mission of eliminating suffering and death due to cancer by 2015—a goal put forth by Dr. von Eschenbach. During his visit, several CCR investigators had an opportunity to discuss their progress in four specific areas: 1) live cell and genome imaging, 2) host genetics, inflammation, and therapy, 3) drug discovery, and 4) lymphoma diagnosis and treatment. A brief overview of the research presented during Dr. von Eschenbach's visit follows.

Live Cell and Genome Imaging

Gordon Hager, PhD, of the Laboratory of Receptor Biology and Gene Expression, described recent developments in real-time live-cell imaging using a fluorescent molecule tag to visualize the location and movement of proteins within a living cell. Real-time imaging has proven to be an amazingly powerful tool for studying the proteins of interest to Dr. Hager's lab: those that interact with chromatin and DNA to regulate gene expression. Using a technique called fluorescence recovery after photobleaching (FRAP), Dr. Hager was able to watch as tagged glucocorticoid receptor (GR) molecules interacted with their target DNA regulatory sites (Figure 1) and show that this lasted for only a few seconds, overturning a 40-year-old central tenet that these

steroid receptors bind statically for extended periods as part of large, multi-protein transcription factor complexes. Dr. Hager concluded from these experiments that nuclear receptors are highly mobile on their gene targets, both *in vitro* and *in vivo*, calling this the "hit-and-run" hypothesis. GR interacts transiently and periodically with hormone-response elements during chromatin remodeling. By testing the inducibility of 30,000 mouse genes using microarrays and a dominant-negative mutant for remodeling protein, Dr. Hager—in collaboration with Paul Meltzer, MD, PhD, of the National Human Genome Research Institute—established that chromatin remodeling is a common feature central to regulation by nuclear receptors. The dynamic interaction of these molecules with their

targets is also strongly ligand dependent, a critical observation because nuclear receptors are major targets in cancer therapy.

Tom Misteli, PhD, of the Laboratory of Receptor Biology and Gene Expression, presented his ongoing work on the application of imaging techniques to study the human genome. The basis of this approach is the fundamental property of genomes that chromosomes, as well as single genes, occupy preferential positions within the nucleus; for example, in human lymphocyte nuclei, chromosome 18 is generally located at the periphery. There is good evidence that these positioning patterns are functionally relevant, and they are known to change during differentiation and development. Dr. Misteli's laboratory has been able to show that nonrandom spatial proximity of genes facilitates the formation of the translocations in Burkitt's lymphoma and other tumors, and his group is conducting experiments to determine positioning patterns during tumor formation and progression. To map and compare positioning patterns of multiple genes and chromosomes more efficiently and accurately, Dr. Misteli is using NCI's extensive imaging and bioinformatics resources to develop a high-throughput automated image acquisition system. The objective is to acquire a large number of highly accurate genome images of normal and tumor cells. Genome positioning data will be combined with microarray data to correlate positioning with gene expression in normal, premalignant, and metastatic cells, as well as cancer stem cells. The findings can be applied to both basic discovery and potential diagnostic applications.

Host Genetics, Inflammation, and Therapy

Kent Hunter, PhD, of the Laboratory of Population Genetics, presented work on the characterization of metastasis susceptibility genes, an area that may explain the seemingly random appearances of metastases in the human population. Crossing transgenic mice that develop mammary tumors and pulmonary metastases with several normal inbred strains resulted in progeny with differing propensities to develop metastatic tumors. Analysis of high and low metasta-

tic strains identified a polymorphism in the signal transduction protein Sip1; inhibiting Sip1 expression significantly reduced the incidence of metastasis in these mice, whereas its overexpression dramatically increased the ability of the tumors to metastasize. These results suggest that modulating the amount of Sip1 in human tumor cells might have therapeutic effects. In collaboration with the research group of Hoda Anton-Culver, PhD, at the University of California at Irvine, Dr. Hunter identified Sip1 polymorphisms associated with the development of human breast cancer. Because genetic polymorphisms are present in every cell in the body, Dr. Hunter is focusing on prospectively identifying patients at high risk for metastasis at, or potentially before, primary diagnosis. Using saliva from mouse models as a surrogate, it has been possible to predict with high sensitivity and specificity which animals will develop highly metastatic disease. Dr. Hunter concluded by mentioning that his lab is working to identify metastasis chemoprevention agents.

Lalage Wakefield, DPhil, of the Laboratory of Cell Regulation and Carcinogenesis, discussed the development of transforming growth factor- β (TGF- β) antagonists as novel therapeutic agents, noting that the NIH has more TGF- β expertise than any other institution in the world. Research conducted in her lab has shown that TGF- β switches from acting as a tumor suppressor early in cancer progression to functioning as a prometastatic factor later in the course of the disease. Treating transgenic mice that develop metastatic breast cancer with antibody-like TGF- β antagonists resulted in a significant reduction in the incidence of metastasis (Figure 2) without affecting primary tumor formation or causing any toxicity that might be expected from inhibiting TGF- β , such

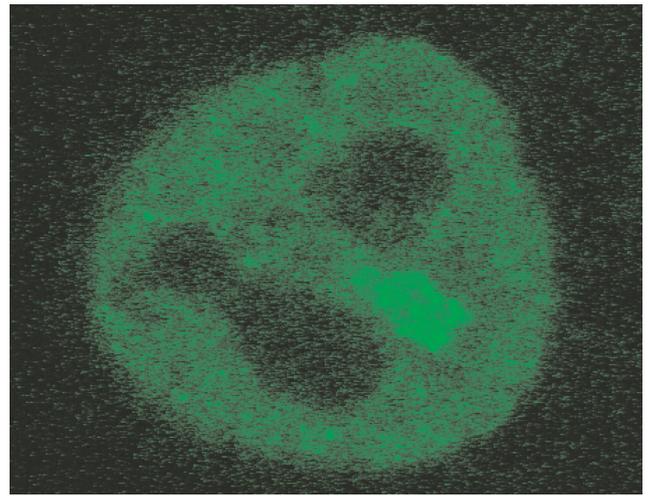


Figure 1. Green fluorescent protein (GFP)–glucocorticoid receptor bound to amplified promoter array: The interaction of GFP-tagged nuclear receptors can be observed on amplified natural gene targets in living cells. (Figure and legend courtesy of Gordon Hager, PhD)

as autoimmunity, inflammation, or enhanced spontaneous tumorigenesis in other organs. Dr. Wakefield's mechanistic analysis demonstrated that TGF- β antagonists act by enhancing endogenous immune surveillance against the tumor. TGF- β antagonists potentially could be combined with other therapeutic modalities to enhance their efficacy; for example, TGF- β seems to be largely responsible for fibrosis following radiation therapy. To facilitate preclinical development of TGF- β -antagonist therapeutic antibodies, Dr. Wakefield and colleagues developed a multi-investigator cooperative research and development agreement (CRADA) with Genzyme Corporation that will also support clinical trials to be conducted at the NCI.

John Letterio, MD, also of the Laboratory of Cell Regulation and Carcinogenesis, described the research conducted in his laboratory through which he and other investigators are evaluating how alterations in signaling through the TGF- β pathway influence carcinogenesis. His lab has developed *in vivo* models in which components of the TGF- β pathway have been disrupted specifically in immune cells. His group is also developing and evaluating new therapeutic agents that target this pathway. Dr. Letterio described studies characterizing the TGF- β receptor-activated Smad3 protein as a key effector of the response to TGF- β in normal

T cells, and as an important suppressor of leukemogenesis in humans. Smad3 expression is lost in leukemic cells of patients with pediatric acute T-cell lymphoblastic leukemia (T-ALL). Dr. Letterio next discussed an important, recently developed model in which deleting the gene encoding Smad4 specifically in T cells resulted in epithelial carcinomas throughout the alimentary tract (unpublished data). Normal epithelial cells produce TGF- β after exposure to environmental microorganisms; it is suspected that the loss of Smad4-dependent signaling in T cells leads to progressive activation of lymphocytes and production of Th2 cytokines, resulting in epithelial hyperplasia. Finally, Dr. Letterio described the chemopreventive activity of novel synthetic triterpenoids in a mouse model of inflammatory bowel disease that progresses to colon cancer. These agents (synthesized in the lab of NCI's Eminent Scholar, Michael Sporn, MD), along with TGF- β antagonists, have great potential for clinical development.

Drug Discovery

Jeffrey Rubin MD, PhD, of the Laboratory of Cellular and Molecular Biology, who purified keratinocyte growth factor (KGF), summarized his basic and clinical research with this protein. Dr. Rubin explained that KGF, which is derived from mesenchymal cells, is a paracrine mediator of epithelial homeostasis with remarkable cytoprotective effects. Oral mucositis resulting from high-dose chemotherapy and radiation seemed a good setting in which to explore a therapeutic use for KGF. He discussed results from a phase III clinical trial sponsored by Amgen, Inc., in which patients with hematologic malignancies received radiation and chemotherapy followed by autologous peripheral blood progenitor cell transplants. Results showed that the patients who were also treated with KGF had a significant reduction in the incidence and duration of severe oral mucositis. Patients treated with KGF required less pain medication and less total parenteral nutrition to supplement oral intake. There was also a decrease in the incidence of febrile neutropenia, suggesting that reducing the damage to the mucosa decreases the incidence

of infection. The U.S. Food and Drug Administration has approved KGF (palifermin) for this indication; favorable results in ongoing clinical trials in solid tumors could mean that approximately 100,000 patients per year may be treated with KGF. In his concluding remarks, Dr. Rubin noted that by increasing patients' tolerance of aggressive therapies, KGF administration is widening the therapeutic window for existing treatments.

Phillip Dennis, MD, PhD, of the Cancer Therapeutics Branch, presented his work in using the Akt pathway as a target for the prevention and treatment of lung

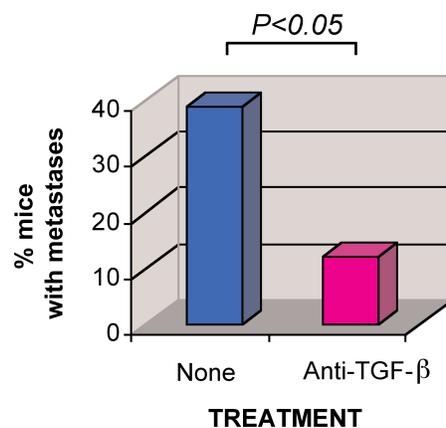


Figure 2. Prolonged treatment with an antibody-like transforming growth factor- β (TGF- β) antagonist can suppress spontaneous metastasis in the MMTV-Neu transgenic mouse model of metastatic breast cancer. (Figure and legend courtesy of Lalage Wakefield, DPhil)

cancer. *Akt* is a proto-oncogene, and its activation is an early event in tobacco-related carcinogenesis. Two tobacco components, nicotine and the tobacco-specific carcinogen NNK, activate the Akt pathway in normal human lung epithelial cells, resulting in partial cell transformation and an altered apoptotic threshold. Cells that survive with DNA damage can ultimately develop into lung tumors. These studies have prompted extramural investigators to include Akt activation as a molecular end point in lung cancer prevention trials. Studies using tissue microarray analysis revealed that Akt was active (i.e., phosphorylated) in more than half of the different tumor types tested and that activation confers a poor prognosis in patients with stage I

non-small cell lung cancer, a finding of vital importance, as increasing numbers of patients are being diagnosed with this disease in screening protocols. Patients diagnosed with tumors with active Akt can thus be stratified to receive more aggressive treatment. Dr. Dennis is also developing new Akt pathway inhibitors—such as several phosphatidylinositol ether lipid analogs (PIAs)—and is testing as potential Akt pathway inhibitors drugs previously approved for other indications. To validate the Akt pathway as a molecular target, clinical trials with Akt pathway inhibitors are being planned at the NCI in patients who have tumors in which Akt is highly active.

Lymphoma Diagnosis and Treatment

Louis Staudt, MD, PhD, of the Metabolism Branch, provided an overview of his work on the molecular diagnosis of lymphoid malignancies. His group has developed an accurate and reproducible single DNA microarray that can provide diagnostic and prognostic information for patients with cancer. Dr. Staudt and colleagues have also developed molecular predictors of outcome—length of survival or response to therapy—in diffuse lymphoma, mantle-cell lymphoma, follicular lymphoma, and chronic lymphocytic leukemia, based on gene expression profiling of diagnostic biopsies. This technology was also used to determine that diffuse large B-cell lymphomas (DLBCL) can be subclassified into three molecularly and clinically distinct diseases: germinal center B-cell-like (GCB) DLBCL, activated B-cell-like (ABC) DLBCL, and primary mediastinal B-cell lymphoma (PMBL). These subtypes arise from B cells at different stages of differentiation, use different oncogenic pathways, and exhibit distinct survival rates following treatment. The discovery of the lymphoma subtypes has also led to the identification of new molecular targets. Dr. Staudt's group is currently attempting to identify additional molecular targets in lymphomas.

The clinical application of Dr. Staudt's research was nicely illustrated by Wyndham Wilson, MD, PhD, of the Experimental Transplantation and Immunology Branch, who described targeted therapeutic approaches for lymphoma. With the

addition of rituximab to standard therapy with cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP), the cure rate for DLBCL was raised from approximately 30% to 50%. To possibly increase the cure rate for DLBCL further, Dr. Wilson developed a novel, continuous-infusion regimen called Dose-Adjusted (DA)-EPOCH, based on a rational design that included optimizing the dosing schedule and pharmacokinetics through application of pharmacodynamic dose adjustment. In contrast to standard therapy with CHOP, this new regimen has proven to be effective against rapidly proliferating tumors. Dr. Wilson's group has shown that the addition of rituximab to DA-EPOCH appears to overcome ABC DLBCL resistance, and preliminary results suggest that the cure of DLBCL has been increased from approximately 33% with CHOP to approximately 75% with DA-EPOCH-rituximab.

Elaine Jaffe, MD, of the Laboratory of Pathology, highlighted the role of the pathologist in lymphoma research, stating that "most of the insights into the pathogenesis of malignant lymphoma have followed on the heels of an accurate pathological description." Knowledge of the pathogenesis of a disease (its molecular pathways) leads to the development of new diagnostic tools, and these tools help to further characterize the disease. This conceptual framework formed the

basis for the development of the Revised European American Lymphoma (REAL) classification and of its descendant, the World Health Organization (WHO) classification—the first internationally accepted classification of lymphomas and leukemias. Dr. Jaffe was a contributor to both systems. Studies by Dr. Jaffe with follicular lymphoma (FL) show how pathogenetic insights can proceed from what is essentially a morphologic observation. She identified an *in situ* form of FL involving germinal centers. Her studies suggest that FL may arise from mature B cells in the germinal centers and not from pre-B cells in the bone marrow. Recent studies using laser-capture microdissection and reverse-phase protein microarray were conducted to compare the apoptotic pathways in Bcl-2-positive and Bcl-2-negative FL. Results show that Bcl_{xL}, an anti-apoptotic protein, was increased in Bcl-2-negative FL. The apoptotic pathways were otherwise very similar between the two FL groups, suggesting that all FLs use a common biochemical pathway.

Major Contributions

Dr. von Eschenbach's visit provided an excellent opportunity to exchange ideas and discuss future directions. He asked insightful and thought-provoking questions that stimulated an excellent dialogue. The science that was presented clearly demonstrated how many results

from intramural research are advancing NCI's mission of eliminating death and suffering due to cancer. Imaging approaches are rapidly advancing our knowledge of fundamental cellular processes—knowledge that will be critical for preventing, diagnosing, detecting, and treating cancer. Furthermore, numerous molecular targets and drugs for those targets are being identified for either prevention or treatment of cancer, and critical targets are being recognized that have the potential for reducing cancer treatment-associated pain and suffering in thousands of individuals. Finally, this work is being conducted by numerous individuals who build on and extend the successes of their intramural colleagues and the extramural research community. The end result is outstanding science and innovative clinical approaches that are providing new hope for cancer patients and their families.

■ **Robert H. Wiltrott, PhD**
Director

Special thanks to L. Michelle Bennett, PhD; Jackie Lavigne, PhD, MPH; Gordon Hager, PhD; Tom Misteli, PhD; Kent Hunter, PhD; Lalage Wakefield, DPhil; John Letterio, MD; Jeffrey Rubin MD, PhD; Phillip Dennis, MD, PhD; Louis Staudt, MD, PhD; Wyndham Wilson, MD, PhD; and Elaine Jaffe, MD

■ BIOTECHNOLOGY RESOURCES

The Genome Analysis Unit

The recent, explosive growth of genomic and proteomic data has dramatically changed the face of biomedical research. The formal scientific discipline of bioinformatics has emerged to address the formidable challenges associated with storing, analyzing, and integrating genetic and other biological information through computer technology. While opening many doors for new areas of investigation, bioinformatics and its associated deluge of data also present many new challenges—challenges that

bench researchers are often ill equipped to face. Many bioinformatics approaches are now necessary components of modern molecular biology research, and just as changes in sequencing technology mobilized the field of molecular biology to move more away from sequencing a single gene to sequencing an entire genome, so bioinformatics is now more frequently being used to address issues involving large classes or families of genes rather than single genes or proteins.

At the CCR, the Genome Analysis Unit (GAU) was created to address some of the issues resulting from the explosive growth in genomic data and to provide a central resource to enhance the research productivity of CCR scientists. The GAU serves this function through a variety of avenues, such as collaborative projects, developing general-purpose web tools, and presenting and organizing training seminars. This article focuses on two of its projects: 1) the NCI Bioinformatics Community Resource, which is a rating

guide for using particular web-based tools, and 2) a collaborative project in which bioinformatics was used to help bench researchers locate a small non-coding regulatory RNA.

The NCI Bioinformatics Community Resource

While the Internet has provided researchers with unprecedented access to repositories of data, literature, analysis tools, and other assorted research information, making effective use of these tools is far from straightforward. In fact, “information overload” is a major problem. Simply keeping track of all the resources can be a full-time job. For example, “The Molecular Biology Database Collection” (*Nucleic Acids Res*, 2005, vol. 33 [Database issue]: D5–D24) listed no fewer than 719 different databases ranging from the well-known databases, such as GenBank (an annotated collection of all publicly available nucleotide and protein sequences), to lesser-known systems such as the Aptamer database (a collection of small RNA or DNA molecules capable of binding ligands, ranging from small organic compounds to whole organisms)! Not only is the number of databases overwhelming, but making efficient and effective use of them is made more difficult by the fact that different viewing and analysis tools may exist at different sites. To address this issue, the GAU has recently launched the NCI Bioinformatics Community Resource (NBCR) (<http://genome.nci.nih.gov/nbcr>). The NBCR is a repository (database) of links to an array of bioinformatics resources useful in the analysis of DNA and protein sequence data. Designed to be a community-managed resource, researchers are invited to provide meaningful reviews of the listed sites and suggestions for new sites. The goal is to construct a rating guide via peer review of those resources that may prove valuable to the NCI community and to provide direction on how best to navigate the ever-growing sea of information associated with those resources. We expect that the NBCR will be a uniquely valuable tool via a rating scheme that reflects “real-world” utility.

Collaborative Research and Custom Tool Development

During the past year, the GAU has been involved in a number of successful collaborative projects with several CCR scientists. The focus of these projects has included custom oligo design for microarray chip production, development of simple web-based tools for identification and extraction of promoter regions, development of gene annotation and DNA codon modification tools, a genome-wide analysis of human promoter regions, and the search for small regulatory RNA (sRNA) candidates in both eukaryotic and prokaryotic organisms. These collaborations have been quite successful in moving NCI science ahead. One such collaborative project is detailed below.

Identification of Tandem Duplicate Regulatory Small RNAs in *Pseudomonas aeruginosa* Involved in Iron Homeostasis

Wilderman PJ*, Sowa NA‡, FitzGerald DJ‡, FitzGerald PC‡, Gottesman S‡, Ochsner UA*, and Vasil ML*. *Proc Natl Acad Sci U S A* 101: 9792–7, 2004.

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Small non-coding RNAs (sRNA) are located predominantly in the intergenic (IG) regions of bacterial genomes. One of the challenges in understanding their contribution to gene regulation has been simply locating them. Previously, Massé and Gottesman 2002 (*Proc Natl Acad Sci U S A* 99: 4620–5) demonstrated that the expression of a Fur-regulated sRNA (RyhB) is responsible for the regulation of an assortment of genes in *Escherichia coli* that are expressed under iron-replete conditions. Sequence homologs of these sRNAs were also identified in other Enterobacteriaceae (e.g., *Salmonella*, *Klebsiella*, and *Shigella*). However, this sequence homology did not extend to the genus *Pseudomonas*. Because the vast majority of the sRNAs that have been described in *E. coli* are encoded in IG regions and no microarray chips are available that cover the IGs of *Pseudomonas*, a

different approach was needed. Thus, a RyhB functional homolog was sought by querying all the IG regions, derived from the whole genome sequence (GenBank id: NC_002516), of the PAO1 strain of *P. aeruginosa* for two predicted properties of such a functional homolog: regulation by a Fur box, and a rho-independent transcription terminator. This analysis yielded only three candidates. Two of the candidates (now termed PrrF1 and PrrF2) were located in tandem between the genes *PA4704* and *phuW* (*PA4705*). Microarray and expression studies, as well as gene deletion experiments, demonstrated that both members of this tandem pair are Fur- and iron-regulated, and that they are functional, but not sequence, homologs of RyhB. Moreover, while homology searches found two putative *prfF* sequence homologs in *P. putida*, *P. fluorescens*, and *P. syringae*, they are considerably distal to each other in these organisms in contrast to their tandem location in *P. aeruginosa*. We conclude from this study that this type of bioinformatics approach is likely to be successful in finding other sRNAs regulated by any well-defined regulatory protein in any sequenced organism that is known to use rho-independent terminators.

In conclusion, the above-mentioned project is just one example where, by bridging the gap between molecular biology and bioinformatics, the GAU has collaborated with CCR scientists to produce a successful outcome not as easily achieved by either partner alone.

NCI scientists wishing to contact the GAU can do so by sending an email to pcf@helix.nih.gov or by visiting our web site at <http://genome.nci.nih.gov/>.

■ Peter FitzGerald, PhD

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Wiggling and Jiggling Can Increase the Effectiveness of AIDS Drugs

Janssen PAJ, Lewi PJ, Arnold E, Daeyaert F, de Jonge M, Heeres J, Koymans L, Vinkers M, Guillemont J, Pasquier E, Kukla M, Ludovici D, Andries K, de Béthune M-P, Pauwels R, Das K, Clark AD Jr, Volovik Frenkel Y, Hughes SH, Medaer B, De Knaep F, Bohets H, De Clerck F, Lampo A, Williams P, and Stoffels P. In search of a novel anti-HIV drug: multidisciplinary coordination in the discovery of 4-[[4-[[4-[(1E)-2-cyanoethenyl]-2,6-dimethylphenyl]amino]-2-pyrimidinyl]amino]benzotrile (R278474, rilpivirine). *J Med Chem* 48: 1901–9, 2005.

Das K, Clark AD Jr, Lewi PJ, Heeres J, de Jonge MR, Koymans LMH, Vinkers HM, Daeyaert F, Ludovici DW, Kukla MJ, De Corte B, Kavash RW, Ho CY, Ye H, Lichtenstein MA, Andries K, Pauwels R, de Béthune M-P, Boyer PL, Clark P, Hughes SH, Janssen PAJ, and Arnold E. Roles of conformational and positional adaptability in structure-based design of TMC125-R165335 (etravirine) and related non-nucleoside reverse transcriptase inhibitors that are highly potent and effective against wild-type and drug-resistant HIV-1 variants. *J Med Chem* 47: 2550–60, 2004.

Lewi PJ, de Jonge M, Daeyaert F, Koymans L, Vinkers M, Heeres J, Janssen PA, Arnold E, Das K, Clark AD Jr, Hughes SH, Boyer PL, de Béthune MP, Pauwels R, Andries K, Kukla M, Ludovici D, De Corte B, Kavash R, and Ho C. On the detection of multiple-binding modes of ligands to proteins, from biological, structural, and modeling data. *J Comput Aided Mol Des* 17: 129–34, 2003.

Although great progress has been made in the treatment of HIV-1/AIDS, treatment failures still occur. One of the primary causes of HIV-1 treatment failure is the emergence of drug-resistant viral variants. Because HIV-1 evolves so rapidly in patients, finding drugs that can consistently hit this “moving target” is a major challenge.

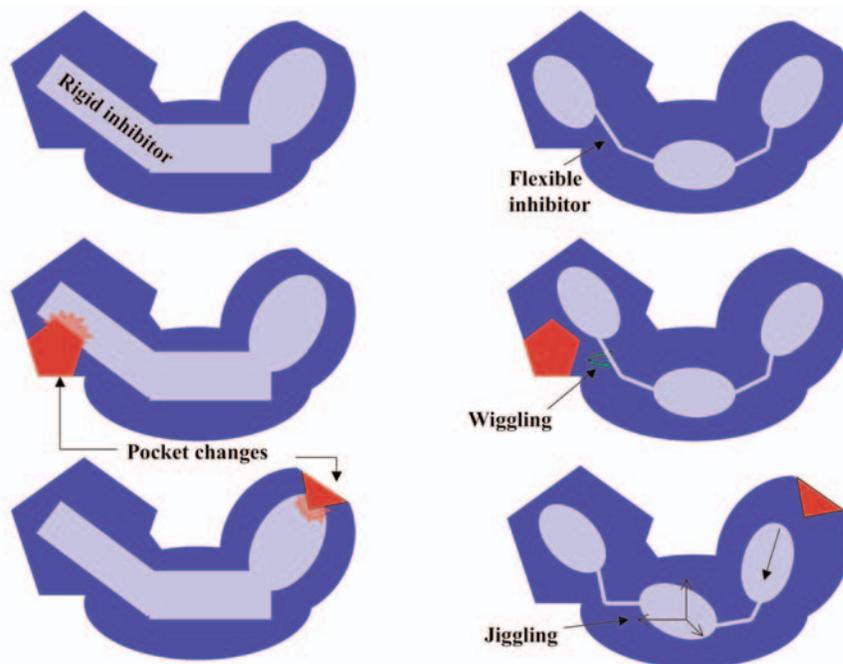


Figure 1. Wiggling and jiggling allow a nonnucleoside reverse transcriptase inhibitor (NNRTI) to effectively inhibit drug-resistant HIV-1 reverse transcriptase. The figure shows, in cartoon form, a comparison of the binding of a rigid inhibitor that cannot reposition itself in the drug-binding pocket and a flexible inhibitor that can reposition itself. Mutations in the drug-binding pocket change the shape of the pocket, causing steric hindrance that interferes with the binding of the rigid inhibitor (left side of the cartoon). An inhibitor that can adapt its shape (wiggling) and binding position (jiggling) in response to the changes in the binding pocket can still bind effectively to mutant drug-binding pockets (right side).

There are four classes of drugs used to treat HIV-1 infections: fusion inhibitors, protease inhibitors, and two types of drugs that inhibit the viral enzyme reverse transcriptase (RT)—nucleoside analogs and nonnucleoside reverse transcriptase inhibitors (NNRTIs). Unfortunately, HIV-1 can become resistant to all the available drugs. However, it is more difficult for the virus to develop resistance to some drugs than to others. One of the critical goals in combating treatment failure is to develop new drugs that will present the greatest possible challenge to viral resistance. When new drugs are developed, it is particularly important that they are effective and potent against resistant viruses that already exist. In the case of the NNRTIs, the ability of the drug to bind tightly to both the wild-type and drug-resistant RTs is a key design consideration.

The compound TMC125-R165335 (etravirine) is a very promising NNRTI currently being tested in patients in the United States. Etravirine was developed through a multidisciplinary effort involving chemical synthesis, tests of drug candidates against both wild-type and drug-resistant HIV-1 strains, structure determination by X-ray crystallography, and molecular modeling. Crystal structures were determined for candidate NNRTIs in complexes with both wild-type and drug-resistant forms of RT. The crystal structures provided the templates for extensive molecular modeling that guided the synthesis of new NNRTIs, which led to the discovery of etravirine and related diarylpyrimidine (DAPY) compounds.

In a recent study published in *J Med Chem* (47: 2550–60, 2004), Das and

colleagues described the structural work behind the discovery of the DAPY compounds and discussed how the conformational flexibility of the molecules, together with their ability to reposition themselves within the drug-binding pocket, allows them to bind effectively to, and inhibit, both the wild-type and drug-resistant RTs. These two complementary properties (which the authors describe as “wiggling” and “jiggling”) allow etravirine and other DAPY compounds to bind to the many different forms of the drug-binding pocket that are found in drug-resistant RTs. This recent work is supported by the molecular modeling calculations of Lewi and colleagues (*J Comput Aided Mol Des* 17: 129–34, 2003), which suggested that

NNRTIs with multiple binding modes would be better able to inhibit drug-resistant RTs.

A drug like etravirine, which can wiggle and jiggle, is able to bind to and inhibit mutant forms of RT that can evade a more rigid NNRTI, which cannot effectively reposition itself and bind tightly to the altered drug-binding pockets of drug-resistant RTs (Figure 1). Das and colleagues proposed that the approach of designing drugs that adapt their structure and binding modes to counteract variation in their binding sites should be applicable to other HIV-1 targets, as well as to targets in other rapidly evolving organisms, including other viruses and bacteria.

Even though etravirine is an exceptionally promising drug, the search for new drugs that can be used in the treatment of AIDS (including additional NNRTIs) continues. Another promising NNRTI in the same family as etravirine is R278474, described in the paper by Janssen et al. (*J Med Chem* 48: 1901–9, 2005). Although the testing of this new compound is not complete, the compound has been very potent in short-term phase II clinical trials.

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■ CANCER AND CELL BIOLOGY

p53 Modulation of Homologous Recombination

Linke SP, Sengupta S, Khabie N, Jeffries BA, Buchhop S, Miska S, Henning W, Pedoux R, Wang XW, Hofseth LJ, Yang Q, Garfield SH, Sturzbecher HW, and Harris CC. p53 interacts with hRAD51 and hRAD54, and directly modulates homologous recombination. *Cancer Res* 63: 2596–605, 2003.

A functional homologous recombination (HR) pathway is essential for faithful genomic replication and cell survival, as unrepaired spontaneous or induced double strand breaks (DSBs) tend to be recombinogenic and/or lethal. However, an overactive HR pathway also could be problematic. For example, inappropriate recombination could lead to losses of heterozygosity, translocations, deletions, or duplications. These processes are all commonly observed in human cancers and tumor cell lines, and elevated levels of RAD51 have been documented in some. This suggests that a balance must be struck in the HR pathway between allowing variability and maintaining genetic stability (Bertrand P et al. *Trends Genet* 20: 235–243, 2004).

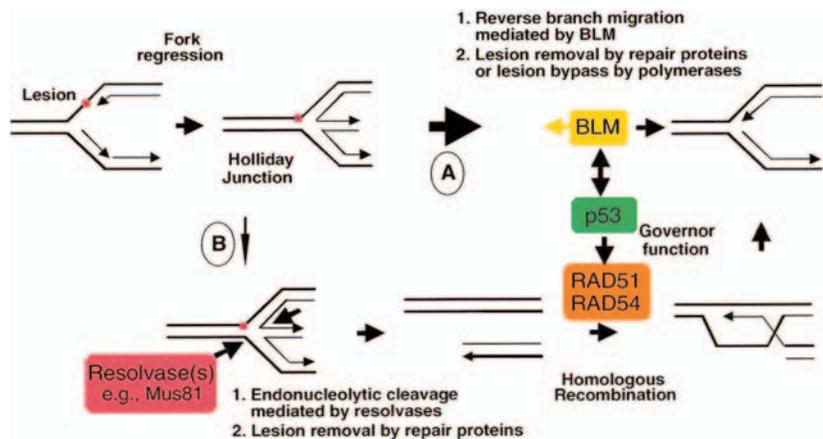


Figure 1. Mechanistic model for the restoration of stalled DNA replication forks. A regressed replication fork is restored by either reverse branch migration, mediated by BLM (Bloom syndrome protein) helicase in a non-recombinogenic pathway (indicated by a thick arrow, pathway A), or a recombinogenic pathway that involves endonucleolytic cleavage by one or more resolvases (e.g., Mus81), followed by RAD51/RAD54-mediated homologous recombination (indicated by a thin arrow, pathway B). p53 functions as a “molecular governor” of homologous recombination. The simplified model shows a single DNA lesion at a replication fork that could represent a carcinogen-DNA adduct, a UV photoproduct, or a base damaged by a free radical.

When p53 tumor suppressor function is compromised, cells exhibit increased rates of spontaneous and induced HR. p53 transcriptional activation is involved in its well-characterized mediation of cell cycle arrest and apoptosis. However, there is evidence for a p53 transcription-

independent function in apoptosis, and p53-dependent inhibition of recombination also appears to be independent of transcription. In fact, p53 is found in complexes with other proteins directly involved in HR processes, including BLM (Bloom syndrome protein), BRCA1,

BRCA2, RAD51, RAD52, RAD54, and WRN (Werner syndrome protein). Our study characterizes p53 interactions with the central HR factors, RAD51 and RAD54.

When cells are challenged with certain DNA damaging agents, probing them for specific repair or checkpoint proteins results in a focal nuclear staining pattern. These foci likely represent sites of damage where the proteins have accumulated. RAD51 accumulates in nuclear foci in cells treated with DSB-inducing agents, such as ionizing radiation or neocarzinostatin, or with agents that stall replication fork progression, such as hydroxyurea. Using an antibody specific for an activated form of p53 phosphorylated at serine-15 (p53pSer15), we found a high percentage of colocalization with RAD51, suggesting that activated p53 accumulated at sites of damage and potential HR repair. The nuclear foci likely represent persistent or slowly repaired breaks, which are prone to inappropriate recombination or misrepair. Thus, p53 accumulation at these sites may be crucial to maintain genetic stability.

We showed that RAD51 coimmunoprecipitated with p53 at endogenous levels in normal human cells to verify that it was directly in the repair complex. Using a mixture of RAD54 antibodies, we also demonstrated that nuclear foci of endogenous RAD54 colocalized with RAD51 and p53pSer15 in normal cells. RAD54 coimmunoprecipitated with both RAD51 and p53 under these conditions.

In addition, we demonstrated that the p53 C-terminus binds to RAD54 *in vitro*. Thus, p53 may be capable of binding directly to both RAD51 and RAD54.

During HR, RAD51 polymerizes on an exposed single strand of DNA from one duplex with the assistance of the chromatin remodeling factor RAD54. The resultant nucleoprotein filament then invades the other duplex. Previous work indicated that p53 could bind to the RAD51 homo-oligomerization domain. When we overexpressed RAD51, we observed complex nuclear networks of high-order RAD51 filaments. However, when p53 was coexpressed with RAD51, formation of these filaments was greatly inhibited. Interestingly, the tumor-derived p53/273H mutant, which inefficiently binds RAD51, did not significantly inhibit polymerization. In addition, polymerization of a p53-binding mutant of RAD51 was not inhibited by p53. These data establish in living cells that p53 can modulate RAD51 polymerization through direct binding.

Finally, we conducted a functional assay looking at the effect of p53 and RAD51 expression on recombination between plasmids, which we first validated with dominant-negative mutants of p53, RAD51, and RAD54. As expected, wild-type RAD51 elevated HR levels. Interestingly, a transcriptionally inactive p53 mutant reduced this back to the basal level. However, the p53-binding mutant of RAD51 was not inhibited by p53.

Cumulatively, the data indicate that p53 inhibits the polymerization of RAD51, thereby inhibiting HR, in a transcription-independent manner.

Other studies from our lab indicate that p53 is transported to sites of potential HR by BLM protein. When replication forks stall, fork regression can occur (Figure 1), and BLM can directly reverse this process in the absence of HR to restore the fork. The presence of p53 may assist by inhibiting both RAD51-mediated fork regression and nucleoprotein filament formation, as well as through an interaction with RAD54. Similar inhibition mechanisms may exist for other modes of HR.

There are some limitations of this study due to the artificiality of the RAD51 polymerization and extrachromosomal HR assay. In addition, controversy remains about whether overactive HR contributes to cancer and whether transcription-independent p53 modulation of HR activity is one of p53's tumor-suppressive functions. Future studies should shed more light on these issues.

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■ TUMOR BIOLOGY/MOLECULAR IMAGING

Lymphatic Drainage Imaging of Breast Cancer in Mice by Micro-Magnetic Resonance Lymphangiography, by Using a Nano-Sized Paramagnetic Contrast Agent

Kobayashi H, Kawamoto S, Sakai Y, Choyke PL, Star RA, Brechbiel MW, Sato N, Tagaya Y, Morris JC, and Waldmann TA. Lymphatic drainage imaging of breast cancer in mice by micro-magnetic resonance lymphangiography using a nano-size paramagnetic contrast agent. *J Natl Cancer Inst* 96: 703–8, 2004.

Breast cancer remains a common malignancy, resulting in approximately 45,000 deaths annually in the United States. Lymph node metastasis has major prognostic implications and is a major criterion used for determining adjuvant therapy requirements. Sentinel lymph node (SLN) biopsy

has become an increasingly popular surgical procedure to assess SLN disease status. Patients with negative biopsy results can be spared the more extensive and traumatic lymph node dissection, which is associated with substantial short- and long-term sequelae. The two most common methods of assessing the SLN are

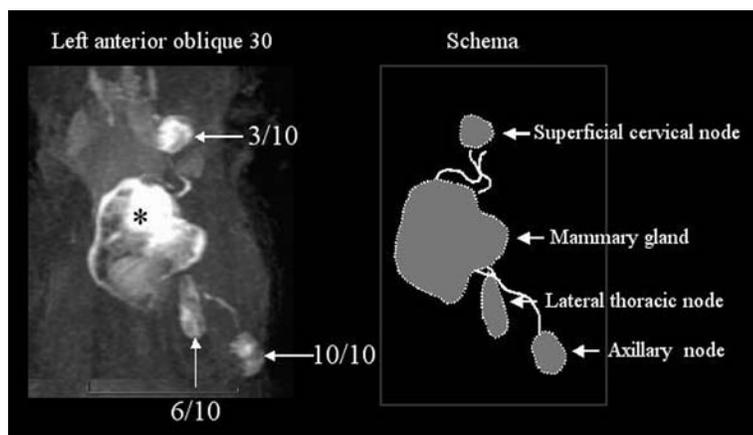


Figure 1. Lymphatic drainage and detected sentinel lymph nodes from the left breast in mice are shown. Detection rates originating from 10 mice for the 3 nodes are indicated.

the use of a blue dye or radioscinigraphy. The former is limited because there is no external means of detecting the node; the latter is limited because of ionizing radiation to the patient, surgeon, and pathologist. Moreover, both methods suffer from poor spatial resolution. Thus, in this study, we focused on the development of an accurate, reliable, and non-invasive magnetic resonance imaging (MRI) method of lymph node assessment.

The use of a macromolecular MRI contrast agent for lymph node assessment is based on a combination of the agent's physical and pharmacokinetic properties including molecular size and shape, lipo/hydrophilicity, charge, a large molar concentration density of Gd(III), and enhanced molar relaxivity conferred by correlation of rotation with a magnetic field. Investigation of these variables has been facilitated by the creation of a library of nano-sized (generations 2–9 [G2–G9]; PAMAM [polyamidoamine] and DAB [polypropyleneimine] dendrimers) MRI contrast agents.

We developed a dynamic micro-MR mammo-lymphangiography method to visualize lymphatic flow from breast tumor tissue to draining lymph nodes to identify an SLN and to determine the presence/absence of nodal involvement. For this study, we chose a G6 PAMAM dendrimer MRI contrast agent (240 kDa) from our library of dendrimer-based, nano-sized contrast agents. Smaller diameter agents (< 4 nm) penetrate

lymphatic capillary membranes and distribute into the interstitial tissue resulting in poor signal-to-background ratios for lymphatic vessels and lymph nodes. Larger molecules (> 13 nm) diffuse slowly and therefore accumulate more slowly in the sentinel nodes necessitating a greater imaging window. We chose the G6 contrast agent (9 nm) to balance these two parameters. That is, it was large enough to be retained, but not too large to inhibit efficient uptake into the lymphatics.

We imaged lymphatic flow from the mammary gland to draining lymph nodes in mice with implanted or spontaneous (Balb/HER2 transgenic) mammary tumors after peritumoral injection of the dendrimer. The method allowed visualization of lymphatic flow from the mammary gland to both metastatic and non-metastatic draining lymph nodes (Figure 1). The U.S. Food and Drug Administration–approved MRI low molecular weight contrast agent, Gd-[DTPA]-dimeglumine (Magnevist), failed to provide an image of the three nodes that were imaged by using the macromolecular agent. This was confirmed by injection of the image-enhancing agent, Magnevist, first, which only vaguely demonstrated the SLN, followed by injection of the G6 in the same mouse, which demonstrated not only the node but also the lymphatic vessel. Additionally, metastatic tumor growth in lymph nodes was detected as “a lack of filling” in the MRI image.

This micro-MRI methodology has advantages over current practice: 1) high spatial resolution (0.1 mm); 2) high time resolution (1 min/frame), enabling differentiation of a sentinel and second lymph node; 3) three-dimensional cine-display from any direction that potentially provides a precise map of the lymph nodes to surgeons; and 4) no adverse effects from either external or internal radiation. Adverse events from MRI contrast agents are related to dose. In this study, G6 was used at 1/2500 of the clinical Magnevist dose (molar concentration) to obtain images, thereby minimizing potential toxicity. Thus, this dynamic micro-MR mammo-lymphangiography method, using a nano-sized MRI contrast agent, may be translatable to clinical practice and should contribute to improved prognosis and quality of life. Current efforts to refine this agent include dual labeling with Gd(III) and an optical imaging element to optimize preoperative and intra-operative visualization of the sentinel node.

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Increased Expression of Dickkopf1 by Palmoplantar Fibroblasts Inhibits Melanocyte Growth and Differentiation

Yamaguchi Y, Itami S, Watabe H, Yasumoto K, Abdel-Malek ZA, Kubo T, Rouzaud F, Tanemura A, Yoshikawa K, and Hearing VJ. Mesenchymal-epithelial interactions in the skin: increased expression of dickkopf1 by palmoplantar fibroblasts inhibits melanocyte growth and differentiation. *J Cell Biol* 165: 275–85, 2004.

Melanocytes, unique cells that specialize in producing the pigmented biopolymer melanin, give rise to visible color in the skin, hair, and eyes. These cells are derived from the neural crest during development (where they are termed melanoblasts): more than 120 genes have been identified that affect pigmentation during the development, migration, survival, and differentiation of these cells. Many of the genes have been cloned and associated with various inherited human pigmentary diseases. (See

<http://ifpcs.med.umn.edu/micemut.htm>.) A number of genes (primarily encoding growth factors and transcription factors) are known to function during melanoblast development and specification, while others (primarily encoding enzymatic and structural components of the pigmentation pathway) are known to function during differentiation.

It is obvious that skin on the palms of the hands and soles of the feet are relatively (often dramatically) less pigmented than the rest of the skin (Figure 1, part A), but to date, no genes have been identified that regulate such differences in pigmentation. This is an interesting and important topic because skin pigmentation is directly, but inversely, related to various types of skin cancer, and darkly pigmented skin is 15 times less susceptible to malignant melanoma as compared with lightly pigmented skin (and 50 times less susceptible to basal and

squamous cell carcinomas). Many clinical terms are used to denote these types of skin, but in this summary, we will use the terms *palm skin* for lightly pigmented, palmoplantar skin and *trunk skin* for the darker, nonpalmoplantar skin.

Our laboratory has been involved in characterizing the regulation of skin pigmentation, resulting in the identification of many physiological and environmental factors (e.g., hormones and UV) that regulate melanocyte growth and differentiation; however, factors that regulate melanocyte density and function in the skin are only poorly understood. An important observation in this regard, made by our collaborators in Osaka, Japan, was that skin transplanted from trunk epidermis to deep wounds in the palm in the same individual gradually assumes the phenotype of the palm skin; that is, it becomes much thicker and much less pigmented, over the course of several months (Figure 1, part B). Indeed, melanocyte density in the trunk skin of Caucasians, Asians, and black/African Americans is virtually identical but is 5-fold lower in palm skin (Figure 1, part C). Since only the epidermis was transplanted, we hypothesized that the relevant regulatory factors were determined by the underlying dermis, which is made up primarily of fibroblasts. A number of recent studies by other groups have shown that different populations of fibroblasts can have widely differing but stable expression patterns of a large number of genes, so it seemed logical that fibroblasts in palm and in trunk skin might produce distinct factors that regulate melanocyte growth and function.

We established primary fibroblasts (derived from the palm and the trunk skin of individual donors) in culture and co-cultured them with melanocytes to recapitulate what was found surgically—that co-culturing with fibroblasts derived from palm skin markedly downregulates

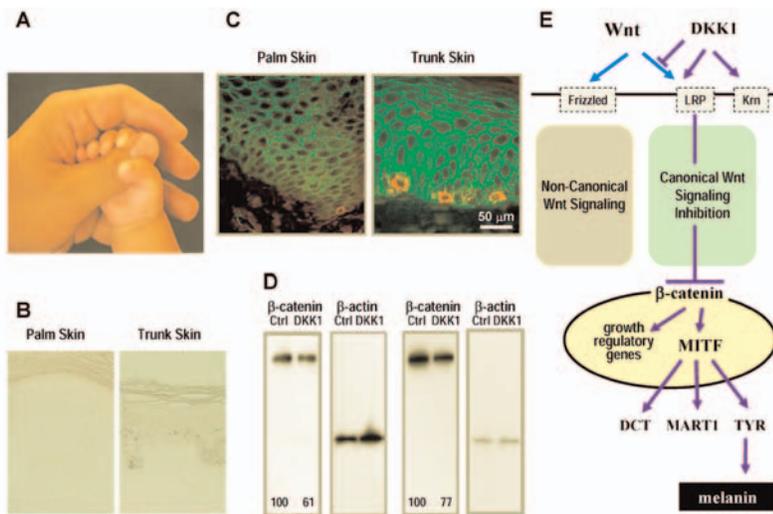


Figure 1. Melanocyte function in palm and trunk skin. *A*) Macroscopic view of hypopigmented palm and hyperpigmented arm skin; *B*) Fontana-Masson staining for melanin showing decreased pigment and increased thickness of palm skin; *C*) Increased expression of β -catenin (green) and melanocytes (stained red due to MART1, a melanosomal protein) in trunk skin; *D*) Expression of β -catenin in melanocytes co-cultured for 5 days with control (Ctrl) or with DKK1-transfected fibroblasts (left-most panel) and in melanocytes treated for 3 hours with or without 50 ng/mL DKK1 per hour (third panel from the left). β -actin is shown as a loading control. (Numbers indicate quantitation of bands.) *E*) Scheme illustrating the possible mechanism by which DKK1 decreases melanocyte growth and differentiation. LRP, Krm, Frizzled: Wnt receptors. MITEF: a melanocyte transcription factor. DCT, MART1, TYR: melanosomal proteins.

melanocyte growth and pigmentation. We used microarray and PCR analyses to examine their gene expression patterns. The majority of genes were comparable between the two populations, but a few genes that were differentially expressed were intriguing, notably, genes relating to two similar factors, dickkopf 1 (DKK1) and DKK3. Not only were they regulated inversely in the two fibroblast populations (from all 5 donors), but the mechanism of action of DKK1 is known to be through Wnt signaling. Wnt signaling has been known for some time to play an important role in regulating melanocyte growth and differentiation, particularly with respect to its effect on melanocyte transcription factors (such as MITF), which in turn regulate melanin production.

A series of molecular and biochemical approaches to stimulate or inhibit DKK1 function were used to observe the effects on melanocyte function. DKK1 (which is preferentially expressed by palm fibroblasts) remarkably inhibited melanocyte growth and also down-regulated pigment production. Inhibiting DKK1 function had the reverse effects, and this proved true whether the DKK1 originated from fibroblasts in co-culture

(Figure 1, part D, left-most panel) or from recombinant protein added to the system (Figure 1, part D, third panel from the left), and the effects could be abrogated by the addition of a DKK1-specific inhibitory antibody (data not shown). As expected, we were able to show that the effects of DKK1 were in fact mediated via Wnt signaling, β -catenin expression, and MITF function. A summary of the DKK1–Wnt– β -catenin–MITF–TYR melanin cascade is shown (Figure 1, part E). Thus, our results provide a basis to explain why skin on the palms and the soles is generally hypopigmented compared with other areas of the body, and might explain why melanocytes stop migrating in the palmo-plantar area during human embryogenesis.

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