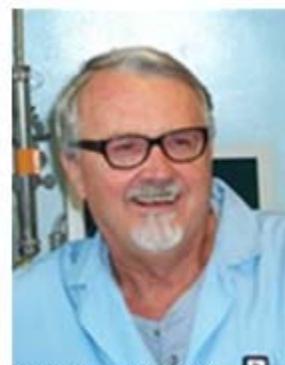


## ■ IN REMEMBRANCE

**In Remembrance: Christopher J. Michejda, PhD**

The CCR research community mourns the loss of Christopher J. Michejda, PhD, whose recent and unexpected death at age 69 has been deeply felt by his friends and colleagues. R. Andrew Byrd, PhD, Chief of CCR's Structural Biophysics Laboratory, expressed the sense of loss felt by the entire community. "Chris Michejda was both a very dear friend and an able scientist. His dedication to research and to NCI was unbounded. He fostered an interdisciplinary approach, as evidenced by all of his collaborations and his willingness to plan and implement joint conferences. He will be missed." CCR

Director Robert Wiltout, PhD, said, "Chris was always generous with his time in support of any project that would make NCI a better scientific community. His standard answer to most requests for help was, 'Okay mate, we'll make it happen.' We'll all miss hearing those words."



Christopher J. Michejda, PhD

An internationally recognized research scientist and head of the Molecular Aspects of Drug Design Section within the Structural Biophysics Laboratory, Chris's initial studies at NCI focused on chemical carcinogenesis and nitrosamines. This seminal work helped to identify the mechanism of activation and metabolic pathways used by these environmental carcinogens to cause cancer. Based on this early work and his expert knowledge of triazene chemistry, Chris and his team developed effective antitumor agents based on triazene derivatives. Eventually, this research led him to an interest in the fundamental problems involved in developing drugs against cancer and viral diseases, such as AIDS.

*A Pioneering Scientist*

Under Chris's direction, his research group at NCI became known for its ability to develop new therapies by combining data from biological studies of disease stages with structural data on potential drug targets within these stages. He pioneered the development of receptor-targeted small molecule toxins that selectively eliminate tumor cells without harming healthy tissue. This approach, now followed by many research labs, has made possible the design of new drugs with better selectivity and low toxicity. Most recently, together with Nadya Tarasova, PhD, Chris discovered a rational approach to specific inhibition of integral membrane proteins that has led to the development of promising novel drug candidates.

The Michejda group's pioneering work with bisimidazoacridones resulted in a new class of compounds potently cytotoxic to liver and pancreatic cancers as well as leukemias. One of these agents is being readied for proposed clinical trials as a treatment for gastrointestinal cancers. Chris's collaborative work with Susan Keay, MD, PhD, of the University of Maryland, resulted in discovery of a so-called antiproliferative factor (APF) in the bladder epithelium of patients who suffer from interstitial cystitis, which unraveled the cause of this disease. By identifying the elements necessary for APF to inhibit normal epithelial growth, the Michejda group paved the way for APF to evolve as a potent inhibitor of bladder and renal cancer. Another collaboration, with Brian Carr, MD, FRCP, PhD, of the University of Pittsburgh, led to the discovery of a new class of highly selective phosphatase inhibitors that are potently active against liver cancer in animal models.

### *An Exceptional Mentor*

Devoted to the broad scientific community, Chris was an exceptional mentor, training many postdoctoral fellows and predoctoral and medical students. The extraordinary breadth of his knowledge ranged from theoretical chemistry to molecular signaling mechanisms. More importantly, Chris had a warm and engaging personality that made all his staff members feel welcome. Susan Holbeck, PhD, a former fellow, recalled, "I met Chris 14 years ago, when I was a postdoc in Frederick, and our interactions continued when I joined DTP (the NCI Developmental Therapeutics Program). His enthusiasm was contagious—it was great fun talking science with Chris. He always put a smile on my face." Another former fellow, Wei Yao, PhD, remembered Chris's kindness. "I first met Chris in 2001 when I was applying for a postdoc position in his lab. That was my first presentation in English in my life. When I struggled to finish all the slides with my broken English and was so nervous, Chris gave me his warm smile and said 'That was great! I can't talk science in Chinese.' I was so lucky to be a member of his lab. He was like a father to me and so many young people from abroad."

One of Chris's long-time colleagues, David Farnsworth, remembered his willingness to discuss science. "Chris was always willing to talk, especially if you had new results to share. He seemed to enjoy most the data that didn't initially make sense or that didn't fit one's hypothesis." Dr. Tarasova said that "Chris was a scientist with a vision and an encyclopedic knowledge in huge areas encompassing organic chemistry and tumor and cell biology. His scientific enthusiasm and energy were infectious as he was trying to make a difference working on novel approaches in anticancer drug development with amazing devotion. And he did make a difference because many of the approaches he suggested turned out to be vastly successful and are very likely to save lives in the near future."

### *A Highly Respected Leader*

Chris published more than 160 articles in prominent scientific journals and held 15 patents for new therapeutic compounds or concepts. He also served as an associate editor for *Cancer Research* and on the editorial boards of *Molecular Cancer Therapeutics*, *Cancer Epidemiology, Biomarkers, & Prevention*, and *Chemical Research in Toxicology*. Highly respected in his field, he was an invited speaker at many local and international conferences

and symposiums. Most recently, Chris played a key role in organizing the joint American Chemical Society and American Association for Cancer Research Conference on Chemistry in Cancer Research, held in February. This meeting featured lectures by prominent leaders in key chemically oriented areas of cancer research, including drug discovery, proteomics, the chemical biology of carcinogenesis, biomarkers and analytical chemistry, modeling and bioinformatics, and structural biology.

As Chair of the Chemistry and Structural Biology Faculty, Chris led CCR's efforts to establish the Program in Interdisciplinary Training in Chemistry for postdoctoral fellows and graduates. He was also an active member of several other NCI faculties and committees and served for many years as a scientific advisor to *CCR Frontiers in Science*.

A close friend and colleague, Larry Keefer, PhD, stated that Chris was "a man whose influence will most assuredly live on. His published research contributions will remain available in libraries and online for all to see. Initiatives he has worked to establish as means to foster interdisciplinary research and to achieve recognition of chemistry's irreplaceable role in the life sciences will continue."

Chris Michejda's scientific innovations, his outstanding mentoring and leadership skills, and his warmth and generosity will always be remembered by his friends and colleagues here at the CCR.

## ■ FROM THE DIRECTOR

**High Risk, High Rewards: The 2007 NCI Director's Innovation Awards**

The 2007 NCI Director's Innovation Awards, presented at the recent Intramural Scientific Retreat, demonstrate once again the breadth and depth of CCR's research program.

These awards recognize and support those scientists who are exploring novel, high-risk research projects that, if successful, could have a substantial impact on their respective fields. First awarded in 2006, these awards provide additional funding for a scientist to pursue a new idea or technique that he/she has developed into a thoughtful, well-designed research proposal. This highly competitive process involves conceptualizing, justifying, and planning a study that is focused on 1) the development of a new approach or technique, or 2) the novel application of an existing approach/technique to a highly significant and/or difficult problem in cancer research. Each proposal is evaluated by a review panel of experts. This year's Principal Investigator (PI) award recipients included five CCR investigators: Steven K. Libutti, MD; Jacek Capala, PhD; Tom Misteli, PhD; Christoph Rader, PhD; and Di Xia, PhD. In addition, 25 other CCR scientists received the Career Development Award. This article highlights the proposals of CCR's PI award recipients.



Robert H. Wiltout, PhD

*Tumor-Targeting Nanoparticles and Radiotherapy: A Promising Combination*

Drs. Libutti (Surgery Branch) and Capala (Radiation Oncology Branch) are a great example of combining complementary strengths. They submitted a winning joint proposal for a study that will explore the use of tumor-targeting nanoparticles to increase the efficacy of radiation therapy for cancer patients.

Colloidal gold is a neutral gold particle synthesized through the combination of gold chloride and sodium citrate. It has been used safely for decades as a therapeutic for patients with arthritis. The particle measures 20–30 nm in diameter and can be linked irreversibly to proteins, peptides, synthetic drugs, and nucleotides. In addition to its properties as a nano-carrier, colloidal gold, being a high-Z element, may also increase the radiation dose delivered specifically to the target cells. TNF $\alpha$ -PEG-colloidal gold (CYT-6091), a recently developed nanoparticle, has been shown to selectively travel to tumor tissue, based on the nanoparticle properties of colloidal gold. Currently, this agent is being evaluated for its ability to selectively deliver TNF $\alpha$  to tumors in a phase 1 clinical trial that is being

conducted by the Surgery Branch's Tumor Angiogenesis Section.

Given the high mass of gold, Drs. Libutti and Capala believe that tumor tissue containing CYT-6091 will receive higher doses of radiation because of the secondary photoelectrons induced in the gold nanoparticles by conventional radiotherapy. The possible synergistic effect of such a combination will be studied *in vitro* using clonogenic survival assays, and *in vivo* using both mouse xenograft and syngeneic tumor models. If successful, this combined approach may result in the ability to lower the external radiation doses necessary to control cancer and, thereby, minimize the side effects while enhancing the benefits of radiation therapy. The potential benefits of this research are significant, both with respect to its therapeutic promise as well as the potential to develop new technologies based on the properties of colloidal gold. In addition, this collaboration between the Surgery Branch, the Radiation Oncology Branch, and an industry partner utilizes the unique environment of the CCR, which fosters innovation and high-risk, yet potentially high-impact translational science.

#### *Aging and Tumor Formation: Investigating the Molecular Link*

Aging is a major cancer risk factor. The innovative proposal by Dr. Misteli (Laboratory of Receptor Biology and Gene Expression) explores basic cell biological discovery to address one of the most pressing issues in cancer research—the mechanisms of age-related tumor formation. It is estimated that by the year 2030, more than 70% of new tumors will occur in individuals 65 years and older. Elucidation of the molecular mechanism involved in physiological aging is critical for advancing our understanding of tumor formation. The naturally occurring premature aging disorders are powerful tools for studying human aging. Dr. Misteli's laboratory has used the premature aging disease Hutchinson-Gilford progeria syndrome (HGPS) as a model for understanding how genome organization contributes to physiological processes, disease, and aging. ([Click here to see his article](#) on this topic in this issue.) HGPS is caused by a single point mutation in the lamin A/C gene, which encodes for one of the key structural proteins of the cell nucleus, and is characterized by numerous nuclear defects, including increased DNA damage and aberrant chromatin organization. The same molecular mechanism responsible for HGPS is also at work during normal aging. Dr. Misteli's award-winning proposal builds on his previous research in this area.

Dr. Misteli and his team will now undertake the first comprehensive investigation of the molecular link between aging and tumor susceptibility. They will systematically compare the gene expression profiles in a collection of tumor-prone and tumor-resistant premature aging disorders. The major premature aging diseases are ideally suited to address this question because they can be sharply classified according to their tumor susceptibility: Bloom syndrome and Werner syndrome's are characterized by dramatically increased tumor susceptibility, whereas Cockayne's syndrome, trichothiodystrophy, and HGPS patients do not develop tumors.

Both conceptually and technically innovative, this genome-wide expression analysis is the first systematic approach toward identifying age-related tumor genes and is also the first transcriptome comparison among premature aging disorders. Candidate genes will be

validated by comparing their expression levels in young and old individuals, and in old individuals with and without a history of cancer. Given that splice variants of the lamin A/C gene are found in HGPS (Scaffidi P and Misteli T. *Science* 312: 1059–63, 2006), these analyses will be complemented by a systematic search for alternative splice variants of 600 cancer-associated genes using LISA (layered and integrated system for splicing annotation) high-throughput RT-PCR analysis developed by a collaborator, Dr. Benoit Chabot (University of Sherbrooke, Québec, Canada). In the long-term, identification of age-related tumor-susceptibility genes will provide the basis for novel diagnostic and therapeutic strategies.

#### *Engineering Hybrid Therapeutics*

Dr. Rader (Experimental Transplantation and Immunology Branch) won an award for his plan to develop an innovative technology that will impact both translational cancer immunotherapy and antibody engineering. This new technology allows for the development of hybrid therapeutics consisting of a small molecule component and antibody component.

In collaboration with Terrence R. Burke, Jr., PhD (Laboratory of Medicinal Chemistry), Dr. Rader's team will generate and evaluate a hybrid therapeutic that targets a cell surface receptor expressed on acute myelogenous leukemia cells with picomolar affinity. Although the focus of Dr. Rader's research is cancer immunotherapy, the generic design of the technology will have broad applicability beyond oncology, particularly in areas with approved monoclonal antibodies, including infectious and autoimmune diseases.

#### *P-glycoprotein: Solving Its Crystal Structure*

Multidrug resistance (MDR) has a profound clinical impact in the treatment of microbial infections, in cancer chemotherapy, and in organ transplantation. Although the mechanisms of MDR appear to be complicated, the expression of P-glycoprotein (P-gp), an integral membrane protein with 12 predicted transmembrane segments, on the surface of a number of cancer cells has been shown to play a role in MDR. Because an understanding of the mechanism of P-gp function requires a detailed three-dimensional (3-D) knowledge of the molecule, a high-resolution crystal structure of P-gp has been sought for many years, yet remains a major challenge. In fact, structures of very few integral membrane proteins have been solved due to two main obstacles: (1) obtaining large amounts of these proteins, and (2) growing crystals suitable for X-ray diffraction experiments. Dr. Xia (Laboratory of Cell Biology) submitted a winning proposal that deals with the second aspect of this problem.

Conformational variability is a unique property essential for the function of biological macromolecules, and visualization of different conformers is critical for understanding the mechanisms by which these proteins function. However, protein mobility is highly undesirable in the process of their structural elucidation by X-ray crystallography, and P-gp appears to be conformationally heterogeneous in solution. Dr. Xia plans to use a number of different conformation-sensitive monoclonal antibodies (mAbs) to select specific conformations of P-gp for crystallization. He believes that the use of mAb fragments will aid crystallization by trapping and stabilizing specific conformers. Monoclonal antibodies have been used before to enhance crystallization, but not to selectively isolate specific

conformers. Dr. Xia and his team plan to use known P-gp mAbs to quantitatively assay their binding to P-gp in the presence or absence of various compounds to select for drugs that specifically enhance the binding of mAb. The resulting P-gp-mAb complex will then be purified and crystallized using methods developed by Dr. Xia's laboratory that have proven successful in obtaining two other membrane protein crystals. Collaborators on this project include Drs. Suresh Ambudkar and Michael Gottesman (Laboratory of Cell Biology) and Dr. Dimiter Dimitrov (CCR Nanobiology Program).

In addition to these four award-winning proposals, 25 other CCR scientists and clinicians received the Career Development Award. Congratulations to Dalit Barkan, PhD; John A. Beutler, PhD; Chi-Ping Day, PhD; Michael G. Espey, PhD; Jeffrey S. Isenberg, MD; Amy Jacobs, PhD; Jonathan L. Jacobs, PhD; Chamelli Jhappan PhD; Andrew Jobson, PhD; Su Young Kim, MD, PhD; Christophe Marchand, PhD; Oyindasola Oyelaran, PhD; Jung-Eun Park, PhD; Jason W. Rausch, PhD; Christophe Redon, PhD; Olga Sedelnikova, PhD; Rosalba Salcedo, PhD; Christina H. Stuelten, PhD; Binwu Tang, PhD; Michael Tangrea, PhD; William Telford, PhD; Masaki Terabe, PhD; Takeshi Tomita, PhD; Tiffany A. Wallace, PhD; and Yili Yang, PhD.

For the complete list of this year's winning proposals, visit [http://ccr.cancer.gov/news/ccr\\_news\\_innovation\\_awards.asp](http://ccr.cancer.gov/news/ccr_news_innovation_awards.asp).

**Robert H. Wiltrott, PhD**

Director

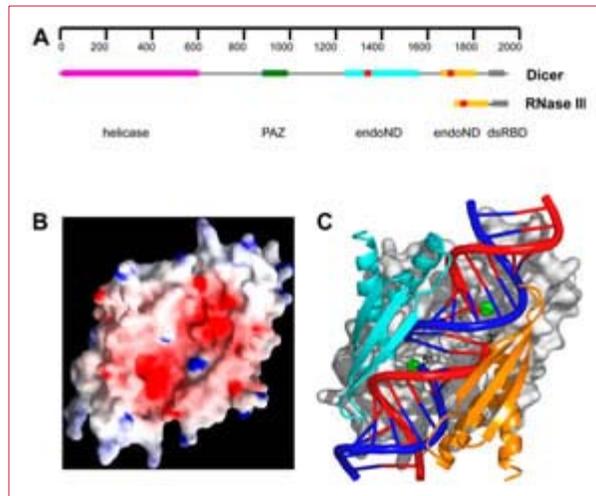
## ■ CELL BIOLOGY

**The Mechanism of Double-stranded RNA Processing by Ribonuclease III: How Dicer Dices**

Gan J, Tropea JE, Austin BP, Court DL, Waugh DS, and Ji X. Structural insight into the mechanism of double-stranded RNA processing by ribonuclease III. *Cell* 124: 355–366, 2006.

**T**he ribonuclease III (RNase III) family is represented by bacterial RNase III and eukaryotic Rnt1p, Drosha, and Dicer, containing approximately 200, 500, 1400, and 1900 amino acid residues, respectively. They are double-stranded (ds) RNA-specific endoribonucleases, characterized by a 9-residue signature motif in their catalytic domains and a 2-nucleotide (nt) 3' overhang in their products. Dicer functions as an RNA-processing enzyme, producing small interfering RNA (siRNA) of approximately 22 nt in length, which mediates RNA interference (RNAi). Bacterial RNase III functions not only as a processing enzyme, but also as a protein that binds dsRNA without cleaving it. *In vitro*, both Dicer and RNase III can be used to produce siRNA cocktails that are effective mediators of gene silencing. Structurally, Dicer is the most complicated member of the family. Bacterial RNase III is much simpler (**Figure 1, part A**) and therefore serves as a model for the entire family.

Bacterial RNase III is composed of an endonuclease domain (endoND) followed by a dsRNA-binding domain (dsRBD). We determined the endoND structure of RNase III in 2001, revealing a symmetric endoND dimer. The dimerization creates a valley that can accommodate a dsRNA substrate. Eight negatively charged side chains are concentrated inside the valley, rendering the valley highly negatively charged (**Figure 1, part B**). Although biochemical data indicate that these residues are involved in the cleavage of dsRNA, we and others in the field were puzzled by the unfavorable interactions between negatively charged dsRNA and negatively charged side chains in the valley until 4 years later when we determined the crystal structure of the first catalytic complex of the entire RNase III family.



**Figure 1.** Structural features of RNase III and Dicer. *A)* Domain structure of *Aquifex aeolicus* RNase III (Aa-RNase III, SWISS-PROT O67082) and *Homo sapiens* Dicer (Hs-Dicer, SWISS-PROT Q9UPY3). Scale on top indicates the lengths of polypeptide chains; boxes in different colors represent individual domains. The red square on the endoND indicates the RNase III signature motif. *B)* Surface representation of the endoND dimer with the colors red and blue indicating negative and positive potential, respectively. *C)* Schematic view of the Aa-RNase III•dsRNA structure. The two endoNDs are shown as a molecular surface; the two dsRBDs are illustrated as ribbon diagrams (helices as spirals,  $\beta$ -strands as arrows, and loops as pipes) and colored in cyan and orange, respectively. The  $Mg^{2+}$  ions are indicated with green spheres; the two RNA strands are shown as tube-and-stick models in blue and red. endoND, endonuclease domain; dsRBD, dsRNA-binding domain; PAZ domain, an RNA-binding module found in Argonaute and some Dicer proteins (initially named for the protein families PIWI, Argonaute, and Zwilli).

The symmetric structure of Aa-RNase III•dsRNA (Figure 1, part C) is composed of two RNase III subunits, two dsRNA molecules, and two  $Mg^{2+}$  ions. The eight negatively charged side chains form the centers of two RNA cleavage sites. In each of the two sites, the 5' phosphate group of the RNA molecule is located in proximity to the  $Mg^{2+}$  ion. The entire structure resembles a clamp that cradles the dsRNA in the midst of the four domains.  $Mg^{2+}$  ions are a key factor in the binding of dsRNA inside the catalytic valley. We have demonstrated that dsRNA is bound outside of the valley if  $Mg^{2+}$  ions are absent. Dicer functions as a monomer. It has two endoNDs and one dsRBD (Figure 1, part A); the two endoNDs form an intramolecular dimer (MacRae IJ et al. *Science* 311: 195–198, 2006). Compared with the dimeric bacterial RNase III, one dsRBD is missing from monomeric Dicer. Studies on a number of dsRBD-containing proteins showed that a single dsRBD is sufficient to provide the proteins with clear specificity for target selection.

Our structure indicates that a single RNA cleavage event occurs on each strand, which creates a terminal phosphate group at the 5' end of the strands, and the two RNA cleavage events together create the 2-nt 3' overhang (Figure 1, part C). The 3'-hydroxyl and 5'-phosphate groups and the 2-nt 3' overhang are hallmarks of RNase III reaction products, which are essential for the incorporation of siRNAs into the RNAi pathway. Underlining the importance of endoND dimerization, the hydrolysis of each RNA strand involves both

endoNDs: Residues from one endoND select the scissile bond, whereas those from the partner endoND carry out the hydrolysis of the phosphodiester bond. The structure reveals a wealth of information about the mechanism of dsRNA processing, which can be extrapolated to other RNase III family members.

**Xinhua Ji, PhD**

Senior Investigator

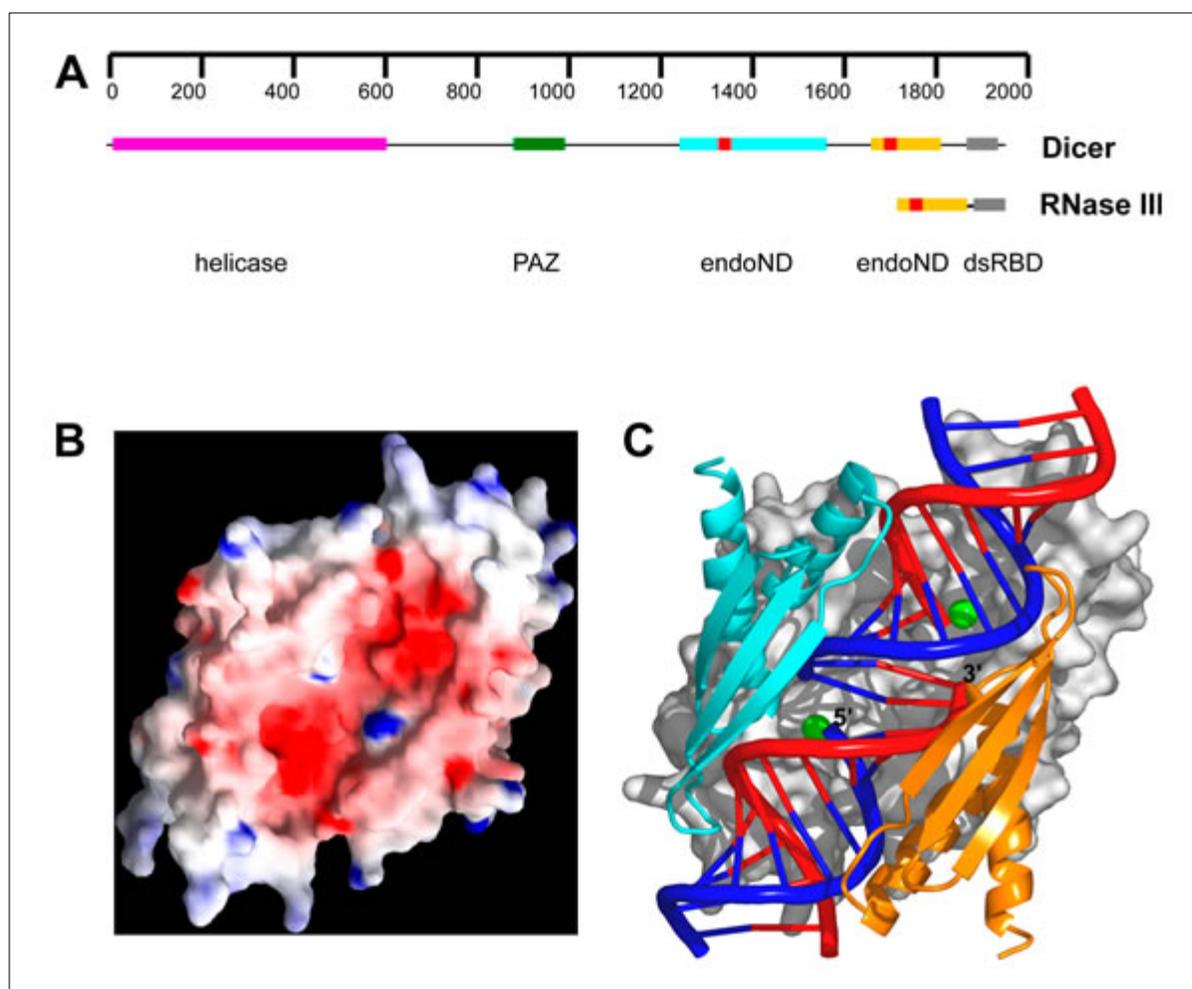
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## ■ CLINICAL RESEARCH

**Effects of Raloxifene on Prolactin and Estradiol Levels in Premenopausal Women**

Faupel-Badger JM, Prindiville SA, Venzon D, Vonderhaar BK, Zujewski JA, and Eng-Wong J. Effects of raloxifene on circulating prolactin and estradiol levels in premenopausal women at high risk for developing breast cancer. *Cancer Epidemiol Biomarkers Prev* 15: 1153–8, 2006.

**R**aloxifene is a selective estrogen receptor modulator (SERM), FDA-approved for the prevention and treatment of osteoporosis. Recently, in the National Surgical Adjuvant Breast and Bowel Project-sponsored Study of Tamoxifen and Raloxifene (STAR) trial, raloxifene was compared with tamoxifen to determine its effect on preventing invasive breast cancer as well as its side effects. Tamoxifen is also a SERM, and the only drug currently FDA-approved for the prevention of breast cancer. The STAR trial showed that both agents reduced invasive breast cancer incidence effectively (by approximately 50%) and that raloxifene treatment was associated with fewer adverse events (Vogel VG et al. *JAMA* 295: 2727–41, 2006). A limitation of the STAR trial is that the study included only postmenopausal women. No long-term studies of raloxifene in premenopausal women have been conducted, yet this agent is of potential interest in this population.

There is a scarcity of knowledge regarding the effects of raloxifene in premenopausal women. Therefore, a phase II trial examining the safety of raloxifene and its effects on bone density, serum hormone levels, and other clinical end points in premenopausal women was led by Jo Anne Zujewski, MD, and Jennifer Eng-Wong, MD, MPH, of the Medical Oncology Branch (MOB/NCI). In this study, premenopausal women at increased risk for breast cancer received raloxifene 60 mg/day for 2 years.

This was the first trial to examine the long-term effects of raloxifene on prolactin, sex hormone-binding globulin (SHBG), and estradiol levels in premenopausal women at increased risk for developing invasive breast cancer. Of the 37 women who enrolled, 27 completed 12 months of raloxifene treatment, with 23 providing paired (baseline and 12-month) serum prolactin measurements and 20 providing paired serum estradiol and SHBG measurements. Prolactin levels did not significantly change with raloxifene treatment, but SHBG levels increased (mean change 7.3 nmol/L;  $P = 0.0001$ ; 95% CI 3.9 to 10.7). Differences between the baseline and 12-month measurements of estradiol were striking when both were taken during the early follicular phase of the menstrual cycle, which was the case for 15 of the 20 women. When the estradiol analysis was restricted to

these 15 women, the mean baseline estradiol level for this group was 87 pg/mL ( $\pm 50$ ), and the levels were elevated after 12 months on raloxifene (mean change 42 pg/mL;  $P = 0.048$ ; 95% CI 1 to 84).

No significant difference was detected between baseline and posttreatment prolactin levels in the only other study to examine levels of prolactin and estradiol in premenopausal women treated with raloxifene; however, there was a 45% increase in estradiol over the entire menstrual cycle for premenopausal women given 200 mg of raloxifene daily for 28 days (Baker VL et al. *J Clin Endocrinol Metab* 83: 6–13, 1998). In our study, estradiol levels increased 48%. Therefore, our prolactin and estradiol results are consistent with this prior report, although women in our study received a lower dose of raloxifene (60 mg daily) and were on the drug for a longer period (12 months).

In conclusion, it is unclear if the increase in both SHBG and estradiol has physiological consequences or how long the elevated levels persist after cessation of raloxifene treatment. No significant change in the circulating levels of prolactin occurred with raloxifene treatment; however, raloxifene may be able to modulate prolactin signaling in breast tissue through mechanisms that are not reflected in a global measurement of circulating prolactin levels. Tamoxifen has been shown to down-regulate prolactin receptor mRNA expression in the breast tissue of postmenopausal women (de Castillo B et al. *Eur J Surg Oncol* 30: 515–9, 2004) and to bind directly to the prolactin receptor and inhibit downstream signaling mediated by prolactin (Biswas R and Vonderhaar BK. *Endocrinology* 128: 532–8, 1991; Das RB et al. *Mol Cell Endocrinol* 98: 1–8, 1993; and Das R and Vonderhaar BK. *Cancer Lett* 116: 41–6, 1997). Raloxifene also may modulate the actions of prolactin locally either by regulating prolactin receptor expression or by inhibiting prolactin receptor signaling in breast tissue. Future research using additional specimens collected during this trial and in collaboration with Barbara Vonderhaar, PhD (Chief of the Mammary Biology and Tumorigenesis Laboratory and Head of the Molecular and Cellular Endocrinology Section) will focus on addressing the effects of raloxifene on the local prolactin/prolactin receptor system in breast tissue.

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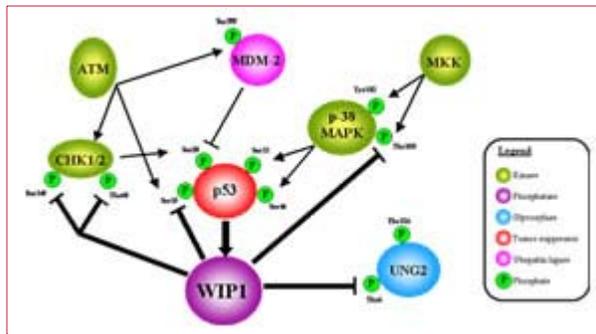
## ■ IMMUNOLOGY

**WIP-ing Down p53 During Thymic Ontogeny**

Schito ML, Demidov ON, Saito S, Ashwell JD, and Appella E. Wip1 phosphatase-deficient mice exhibit defective T cell maturation due to sustained p53 activation. *J Immunol* 176: 4818–25, 2006.

In 1997, we reported the identification of a novel gene transcript whose expression was induced in response to ionizing radiation in a p53-dependent manner, and whose protein product showed homology to the type 2C protein phosphatases. We named the novel gene, wildtype p53-induced phosphatase 1 (*WIP1*), and it was later given the Genebank symbol, *PPM1D* (for protein phosphatase 1D magnesium-dependent, delta isoform). Preliminary results suggested that WIP1 might contribute to growth inhibitory pathways activated in response to DNA damage in a p53-dependent manner.

To investigate possible functions of WIP1, we evaluated its substrate specificity (Figure 1). We first found that WIP1 was able to specifically dephosphorylate the phosphatase p38 MAP kinase at phospho-Thr180, and its overexpression blocked UV-induced p53 activation in cultured human cells. Therefore, this phosphatase, which is induced by p53, can act in a negative feedback mechanism to turn off p53-activating signals. However, phosphatase activity is not usually directed to a single substrate. WIP1 was found to also specifically dephosphorylate the nuclear isoform of uracil DNA glycosylase (UNG2) at phospho-Thr6, leading to suppression of DNA base-excision repair. More recently, WIP1 was reported to dephosphorylate Chk1 at phospho-Ser345, Chk2 at phospho-Thr68, and p53 at phospho-Ser15. Because these signaling molecules activate DNA repair pathways and cell cycle checkpoints to maintain genomic integrity, suppression of these key homeostatic functions may, in part, reflect *WIP1*'s character as an oncogene. WIP1 is amplified or overexpressed in several types of human tumors, including breast tumors, neuroblastomas, and ovarian clear cell adenocarcinomas. In fact, the gene encoding WIP1 (*PPM1D*, at 17q22/q23) is amplified in human breast tumor cell lines and in approximately 11% of primary breast tumors, most of which harbor wild-type p53. This suggests that *PPM1D* overexpression contributes to the development of human cancers by suppressing p53 activation.



**Figure 1.** Substrate specificity of the type 2C protein phosphatase WIP1, which directly or indirectly modulates p53 activity or DNA repair.

To further determine the normal biological function of WIP1 in mammalian organisms, we generated WIP1-deficient mice. These mice were viable but showed a variety of postnatal abnormalities. Notably, mice lacking WIP1 showed increased susceptibility to pathogens and diminished T- and B-cell functionality. Our studies also determined that fewer lymphoid cells were present in the periphery, a finding that could not be accounted for by reduced proliferation or enhanced apoptosis. This prompted us to examine T-cell development in the thymus more closely.

T cells derived from the thymus provide the classical cell-mediated host response that controls intracellular pathogenic organisms. Although many of the players involved in thymic ontogeny have been identified, there still remain large gaps in our knowledge of the molecular pathways involved. In the late 1990s, the potential involvement of the tumor suppressor protein p53 in T-cell development was suggested by the partial rescue of certain immunodeficient mouse models by the elimination of p53. However, because p53-deficient animals exhibited normal T-cell development and were able to mount normal immune responses to pathogens, it was accepted that this tumor suppressor plays no role in T-cell development.

The number of T cells in the thymus of young WIP1-deficient mice is severely reduced, but as the animals age, the normal process of thymic involution does not occur. Therefore, the number of thymocytes in age-matched WIP1-deficient mice approaches that of normal mice as the animals age. Concentrating on young mice, we determined that the loss of T cells was occurring in  $\alpha/\beta$  T cell-receptor cells between the double negative (DN)–to–double positive (DP) transition. Specifically, we observed a block at the last stage (DN4) of development in mice lacking WIP1 that corresponded to the maximal WIP1 mRNA expression found in normal mice. The absence of WIP1 resulted in defective cell cycle progression of the DN4 cells. The few cells that did acquire the DP phenotype were more susceptible to spontaneous apoptosis, possibly because of lower levels of the anti-apoptotic factor Bcl-x<sub>L</sub>. Although cell cycle progression and apoptosis are controlled by a number of mechanisms, both can be controlled by p53. We determined that p53 protein levels, p53 phosphorylation status, and the levels of the downstream effector protein p21 were increased in DN4 and DP cell populations in the absence of WIP1. It is interesting to note that increased p53 activity occurs between the

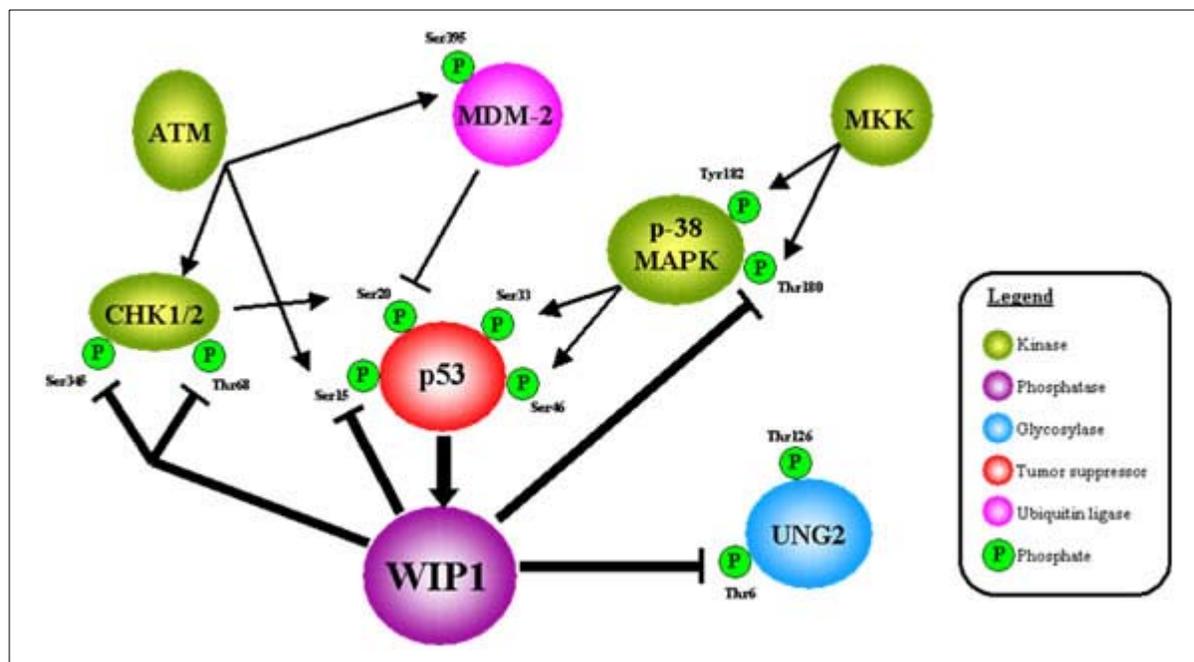
DN-to-DP transition, because some T cell-deficient mice (those with severe combined immune deficiency, *Rag1/2<sup>-/-</sup>*, and *CD3ε<sup>-/-</sup>*), which have a severe defect in the same transition, can be partially rescued by the absence of p53. Because p53 was upregulated during the transition in the absence of WIP1, we crossed WIP1-deficient mice with those that were p53 deficient to determine if T-cell development can be rescued by eliminating p53 activity. Thymic development in these doubly deficient mice was normal, suggesting that elevated levels of p53 were detrimental to α/β T-cell development. Thus, WIP1 phosphatase appears to play an important role in T-cell development by turning off p53 at a key point in thymic development.

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**Figure 1.** Substrate specificity of the type 2C protein phosphatase WIP1, which directly or indirectly modulates p53 activity or DNA repair.



## ■ MOLECULAR BIOLOGY

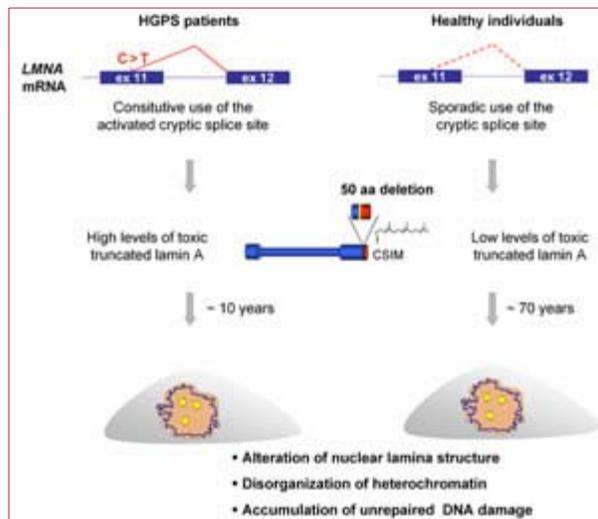
**Aging, Nuclear Architecture, and Cancer**

Scaffidi P and Misteli T. Lamin A-dependent nuclear defects in human aging. *Science* 312: 1059–63, 2006.

**A**ging is a major cancer risk factor. It is estimated that by the year 2030 more than 70% of new tumors will occur in individuals 65 years and older. Elucidation of the molecular mechanism involved in physiological aging is critical for advancing our understanding of tumor formation. However, studying the human aging process at the molecular level is difficult, and it is rapidly becoming clear that animal models do not provide an adequate picture of human aging. Naturally occurring premature aging diseases are powerful tools to explore human aging and its link to cancer.

Possibly the most dramatic and remarkable premature aging disorder is Hutchinson-Gilford Progeria Syndrome (HGPS). Patients appear normal at birth. However, they experience slow physical development within a few months and rapidly develop typical aging symptoms, including loss of hair, changes to their bone structure, loss of subcutaneous fat, and most importantly, the patients are afflicted by atherosclerosis, which is invariably fatal in their mid-teens.

One of the surprising features of HGPS is its molecular basis. The vast majority of cases are caused by a point mutation in the lamin A/C gene, which encodes two of the major architectural proteins of the cell nucleus, lamins A and C. These proteins, together with B-type lamins and numerous lamin-associated proteins, form the nuclear lamina, an interconnected structural meshwork at the periphery of the cell nucleus thought to be involved in protecting the genome from mechanical stress. The detrimental effect of the disease-causing mutation is brought about by virtue of its activation of a cryptic splice site within exon 11 of the lamin A/C gene, leading to the production of a truncated form of lamin A, referred to as progerin, which appears to disrupt the structure and function of the lamina network (**Figure 1**).



**Figure 1.** Molecular parallels between premature aging and physiological aging. The premature aging disease Hutchinson-Gilford Progeria Syndrome (HGPS) is caused by activation of a cryptic splice site in the lamin A/C gene (*LMNA*) leading to the production of a dominant-negative lamin A protein isoform. The protein disrupts nuclear function and leads to defects in chromatin organization and DNA repair. During normal aging, the same cryptic splice site in *LMNA* is used at low levels. Although the production of aberrant lamin A is tolerated in young cells, it leads to defects in aged cells similar to those in the cells of HGPS patients. CSIM, C-terminal CAAX motif (C, cysteine; A, usually an aliphatic residue; and X, any amino acid).

Because the major cellular function of lamin A is to establish nuclear architecture, it was not unexpected that HGPS patients have numerous major defects in nuclear structure. Most importantly, HGPS nuclei have aberrant shapes. Also their chromatin is disorganized, and they contain an increased amount of DNA-damage lesions.

Ever since the discovery of lamin A as the cause of a premature aging disease, a lingering question has been whether defects in nuclear architecture and lamin A in particular were also in any way relevant to physiological aging. This was a particularly pertinent issue because HGPS patients do not exhibit some of the typical hallmarks of aging such as dementia or tumor susceptibility, making the classification of HGPS as a premature aging syndrome somewhat uncertain.

We set out to uncover a potential link between lamin A and normal aging, by asking if cells from old individuals show defects in nuclear structure similar to those found in HGPS cells. Sure enough, we found that cells from 75- to 90-year-old individuals had hallmarks of HGPS patients' cells, including disorganized chromatin and increased levels of unrepaired DNA lesions. These simple observations were the first indication that changes in nuclear architecture are related to the aging process.

But are any of these age-related defects due to the same molecular mechanisms that cause HGPS? We found evidence for such a link when analysis of the splicing pattern of the lamin A/C gene revealed that the same cryptic splice site whose activation causes HGPS is also used at low frequency in healthy individuals and leads to the production of low levels of

progerin (Figure 1).

At the time, we hypothesized that the use of the cryptic splice site would increase with age resulting in elevated production of aberrant protein and thus leading to the observed nuclear defects. To our surprise, this was not the case and no accumulation of progerin was found in aged cells. To prove that the production of the aberrant lamin A protein was indeed responsible for the age-related nuclear defects, we took advantage of technology previously developed by us to block the aberrant splicing event in the lamin A/C gene (Scaffidi P and Misteli T. *Nat Med* 11: 440–5, 2005). To do so, we introduced into cells a morpholino oligonucleotide complementary to the aberrant splice site. The oligonucleotide blocks the access of the pre-mRNA splicing machinery to the cryptic splice site and in this way suppresses the production of progerin mRNA and consequently protein. When applied to cells from old individuals, we found that several age-related nuclear defects were reversed and cells had hallmarks of young cells. Based on these observations, we propose that prolonged exposure to low levels of progerin leads to deleterious effects in aged cells (Figure 1).

These experiments have been insightful for two reasons. First, they document a novel mechanism in aging. Lamin A and nuclear architecture are clearly involved in aging, although it is not clear how they act at the molecular level or what kind of cellular age-related responses they trigger. The fact that we can reverse the cellular aging symptoms by elimination of the aberrant lamin A protein is obviously a tantalizing observation. Second, and maybe more importantly, our results establish HGPS as a true aging model. We are particularly interested in this finding because one of the key features of HGPS is the absence of tumors, whereas most other premature aging diseases are characterized by high tumor susceptibility. We are currently exploring whether HGPS will be a useful model system for delineating the molecular links between aging and tumor formation.

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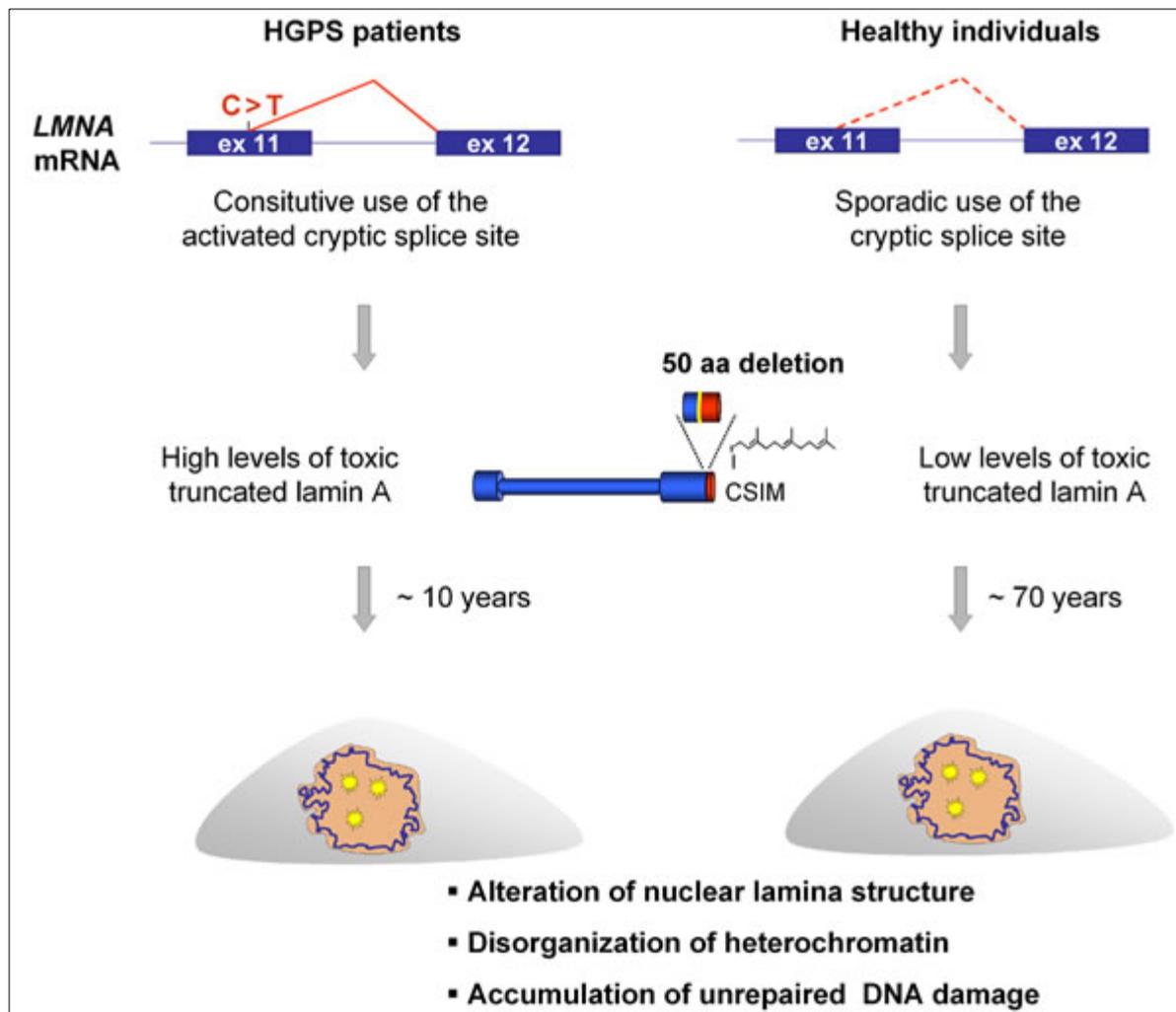
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**Figure 1.** Molecular parallels between premature aging and physiological aging. The premature aging disease Hutchinson-Gilford Progeria Syndrome (HGPS) is caused by activation of a cryptic splice site in the lamin A/C gene (*LMNA*) leading to the production of a dominant-negative lamin A protein isoform. The protein disrupts nuclear function and leads to defects in chromatin organization and DNA repair. During normal aging, the same cryptic splice site in *LMNA* is used at low levels. Although the production of aberrant lamin A is tolerated in young cells, it leads to defects in aged cells similar to those in the cells of HGPS patients. CSIM, C-terminal CAAX motif (C, cysteine; A, usually an aliphatic residue; and X, any amino acid).

## ■ VIROLOGY

**Human Alpha-defensins Block Papillomavirus Infection**

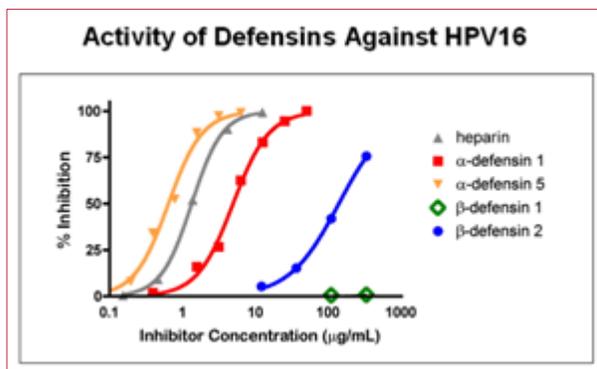
Buck CB, Day PM, Thompson CD, Lubkowski J, Lu W, Lowy DR, and Schiller JT. Human alpha-defensins block papillomavirus infection. *Proc Natl Acad Sci U S A* 103: 1516–21, 2006.

**S**exually transmitted human papillomaviruses (HPVs) are the primary cause of cervical cancer. HPVs have also been implicated in a substantial fraction of other genital cancers, as well as some head, neck, and anal cancers. Although only a minority of infected individuals develop cancer, HPV infection is very common, and cervical cancer kills several hundred thousand women worldwide each year.

Papillomaviruses replicate exclusively in stratified squamous epithelial tissues such as the skin or the genital mucosa. Because the viral life cycle is closely linked to cellular differentiation in these tissues, papillomaviruses cannot be cultured using conventional monolayer cell lines. Recently, we in CCR's Laboratory of Cellular Oncology have developed systems to efficiently mass-produce papillomavirus-based gene delivery vectors, known as papillomavirus pseudoviruses. HPV pseudoviruses, which are capable of efficiently delivering reporter plasmids to a wide variety of cell lines, have rapidly become a useful tool for studying the initial infectious entry phase of the HPV life cycle. The availability of reporter pseudoviruses has made it possible to perform targeted screens to identify compounds that might inhibit the infectious entry of papillomaviruses.

We used HPV pseudoviruses to perform targeted screens of commercially available candidate inhibitor compounds. Initial screens focused on peptide effectors of innate immunity that have previously been found in the human female genital tract. By far, the most promising compounds identified were a group of human innate immune effector peptides known as  $\alpha$ -defensins. Defensins fold into a disulfide-stabilized  $\beta$ -sheet structure that allows them to kill various types of bacteria and viruses. Initially, it was believed that defensins, which have a high cationic charge, acted primarily by disrupting the relatively anionic lipid membranes of various bacteria and lipid-enveloped viruses. More recently, it has been appreciated that another important action of these molecules is to antagonize viruses, such as HIV-1, in ways that may involve signaling or other modifications of host cells. Other recent reports have shown that defensins can block the uptake of bacterial toxins, such as *Anthrax* lethal toxin and *Pseudomonas* exotoxin. Furthermore, some defensins trigger cellular signaling through toll-like receptors, which are normally involved in detecting common molecular patterns displayed by microbial pathogens.

Using pseudoviruses transducing a green fluorescence protein (GFP) gene as a marker of infection, we showed that  $\alpha$ -defensin types 1, 2, 3, and 5 are potent inhibitors of the infectious entry of HPVs into cultured cells (Figure 1). In contrast,  $\beta$ -defensin types 1 and 2 displayed much less or no inhibitory effect against HPV infection. Microscopy studies revealed that  $\alpha$ -defensins block HPV escape from endocytic vesicles but do not interfere with virion binding, internalization, or virion uncoating. Because HPVs have a naked (non-enveloped) surface, the result adds to the growing realization that the antimicrobial power of defensins extends beyond their ability to chemically disrupt microbial membranes.



**Figure 1.** Inhibition of HPV16-GFP (green fluorescent protein–labeled human papillomavirus 16) pseudovirus infection in HeLa cells by human defensins. Pseudovirus and inhibitors were added to subconfluent cells in 96 well plates and GFP-positive cells were counted by a fluorescence-activated cell sorter (FACS) 3 days post infection. Heparin is a highly sulfated form of heparan sulfate previously described as a potent inhibitor of papillomavirus infection.

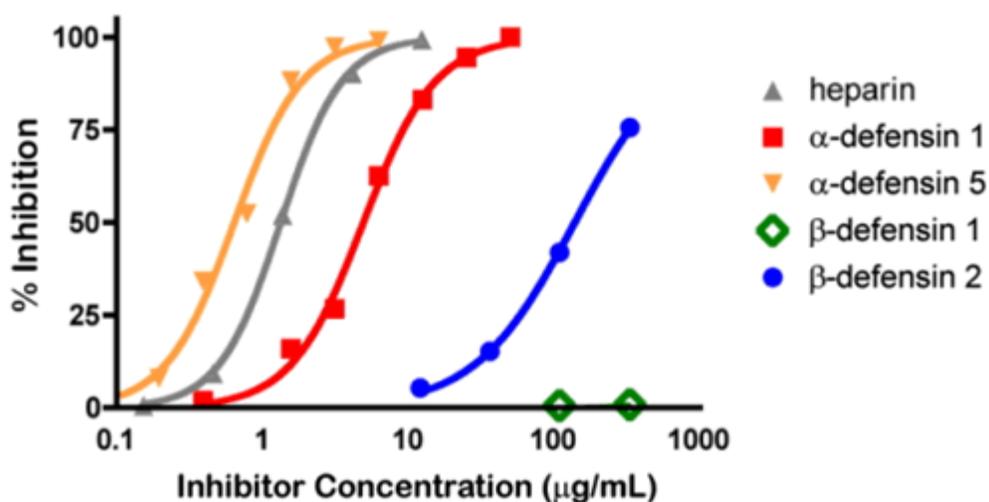
Recent reports have shown that some  $\alpha$ -defensins may be present in the female genital tract at concentrations that inhibit HPV infection *in vitro*. Although the levels of  $\alpha$ -defensins 1 through 3 increase under conditions of inflammation,  $\alpha$ -defensin 5 appears to be produced constitutively at levels near the *in vitro* inhibitor concentrations. The average level of various defensins probably differs between individuals, perhaps reflecting the fact that humans are genetically polymorphic at the level of defensin gene copy number. In one intriguing study, women who appear to exhibit natural resistance to infection with HIV-1 were shown to have consistently higher levels of  $\alpha$ -defensins 1 through 3 in cervical biopsy samples. It will be important to determine whether higher  $\alpha$ -defensin 5 levels correlate with women’s relative resistance to HPV infection.

Recent meta-analyses have concluded that condoms, as routinely used, have limited effectiveness in preventing the transmission of HPVs. Thus, there is significant interest in the development of compounds that might be used as topical microbicides to block the transmission of HPVs and other sexually transmitted infections. The fact that  $\alpha$ -defensins exhibit potent, broad-spectrum antimicrobial activity *in vitro* raises the possibility that they might function to block HPV transmission if applied as topical microbicides. Furthermore, the fact that  $\alpha$ -defensins are ordinarily present in the female genital tract suggests that they would be safe to use for routine topical application.

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### Activity of Defensins Against HPV16



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