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Research Interests

Characterization of signaling cascades that link oncogenic hyperproliferation and activation of tumor suppressor genes/proteins, especially, mechanisms of regulation of the Arf-Mdm2-p53 self-autonomous tumor surveillance pathway. Transgenic/knock-out/knock-in mouse models of lung and breast cancers. Analysis of human lung/breast cancer specimen for alterations for tumor suppressor gene/oncogene candidates and determination of their prognostic values. Use of anti-sense oligonucleotide DNA and shRNA in cancer therapy.

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Education & Training

- M.D., Gifu University School of Medicine, Japan, 1984
- Ph.D., University of Tokyo, Japan, 1990
- Fellowship, Osaka University School of Medicine, Dept. of Medicine III, Japan, 1990-1996
- Associate Investigator, St. Jude Children's Research Hospital (Dept. of Tumor Cell Biology, Dr. C. J. Sherr's laboratory), 1996-2002

In Dr. Sherr's lab (Dept. of Tumor Cell Biology) at St. Jude Children's Research Hospital/HHMI and my own lab at Wake Forest University School of Medicine, I have been working on the signaling pathways that involve the Dmp1 (cyclin D binding myb-like protein 1; Dmtf1) transcription factor that was isolated in a yeast two-hybrid screen with cyclin D2 bait. We reported that gene expression and cell cycle arrest mediated by Dmp1 (Dmp1α) is antagonized by D-type cyclins through a Cdk-independent mechanism (23). The *INK4a/ARF* locus on human chromosome 9p21 is the second most frequently disrupted locus in human cancers, second only to *p53*. This locus regulates both the RB (by $p16^{INK4a}$) and p53 (by $p14^{ARF}$) pathways, and is deeply involved in the control of stem cell-ness in a variety of tissues. We found that Dmp1 directly binds to the *Arf* promoter to activate its gene expression, thereby inducing p53-dependent cell cycle arrest (21). *Dmp1*-deficient mice we created were prone to spontaneous tumor development, which was accelerated when the animals were neonatally treated with ionizing radiation or dimethylbenzanthracene (18, 19).

Although *Dmp1*-knockout mice develop a broad spectrum of epithelial and non-epithelial tumors, lung tumors were the most frequently encountered neoplasms in *Dmp1*-null and *Dmp1*-hetrozygous mice (18, 19). Of note, the wild type *Dmp1* allele was retained and expressed in tumors arising from $Dmp1^{+/-}$ mice, demonstrating a typical haplo-insufficiency of Dmp1 in tumor suppression (book chapter 1). Tumors from *Eμ-Myc*; $Dmp1^{-/-}$ or $Dmp1^{+/-}$ mice rarely showed mutations, deletions, or silencing of $p19^{Arf}$ or *p53*, suggesting that Dmp1 is a critical regulator of the Arf-p53 tumor suppressor pathway *in vivo* (18).

The research in our lab has focused on the regulatory mechanisms of the Dmp1-Arf-p53 pathway, and how it was disrupted in human lung/breast cancers. We found that the *Dmp1* promoter was efficiently activated by oncogenic Ras^{V12} as well as by constitutively active c-Raf, Mek1/2, and Erk1/2 in primary cells (17). $p19^{Arf}$ did not significantly accumulate in response to oncogenic Raf signaling in *Dmp1*-null cells, which were refractory to Raf-mediated cell cycle arrest, suggesting the critical role of Dmp1 as a mediator of oncogenic Ras-Raf-Mek-Erk signaling and the Arf-p53 tumor suppressor pathway (17). Consistently, $K\text{-}ras^{LA}$ lung tumorigenesis was significantly accelerated in both $Dmp1^{+/-}$ and $Dmp1^{-/-}$ mice with larger tumors with invasion/metastasis (11). We also found that loss of heterozygosity (LOH) of h*DMP1* was found in ~35 % of human non-small cell lung carcinomas, especially those that retain wild type *INK4a/ARF* and/or *p53* (11). The *Dmp1* promoter was also activated by an inflammatory cytokine TNFα mediated by NF-κB (12) as well as dsDNA breaks (7) and activated Mekk1 (Fig. 1), indicating that Dmp1 is a mediator of a variety of stress signaling. We conducted GeneChip microarrays using $Dmp1^{+/+}$ and $Dmp1^{-/-}$ lungs and found that other transcriptional targets for Dmp1α include *Areg*, *Thbs1*, *JunB*, and *Egr1* (9), suggesting that it is involved in signal transduction, cell proliferation, angiogenesis, and metastasis. In breast cancer, we characterized the signaling pathway between HER2/neu (or cyclin D1) and Dmp1 using *MMTV-neu* (or *cyclin D1*) mice as a model (3, 4, 8; Fig. 1). Both Dmp1 and p53 were induced in pre-malignant hyperplastic lesions from *MMTV-neu* mice where mammary carcinogenesis was significantly accelerated in both $Dmp1^{+/-}$ and $Dmp1^{-/-}$ mice with more aggressive phenotypes with invasion/metastasis. Tumors from *Dmp1*-deficient mice showed significant downregulation of *Arf* and $p21^{Cip1}$, showing p53 inactivity and more aggressive phenotypes than those from $Dmp1^{+/+}$*;neu* mice (8). Thus, our study shows pivotal roles of *Dmp1* in HER2/neu (cyclin D1) - p53 signaling and breast cancer prevention (3, 4, 8). Of note, our recent study shows that Dmp1α physically interacts with p53 through p53's carboxyl-terminal and Dmp1's DNA-binding domains (7). Dmp1α antagonized p53's ubiquitination by HDM2 both *in vitro* and *in cell* and restored p53's nuclear localization that had been lost with HDM2 expression (7); Dmp1 also stabilized p53 binding to transcriptional target genes (1). Dmp1α-p53 interaction significantly increased the levels of p53 independent of Dmp1's DNA-binding, and hence both $p21^{Cip1}$ and *Bbc3* promoters were synergistically activated by co-expression of Dmp1α and p53 in $p53^{-/-}$; $Arf^{-/-}$ cells (7). In accordance, the induction of $p21^{Cip1}$ and *Bbc3* by genotoxic drug treatment was more seriously affected in $Dmp1^{-/-}$ and $p53^{-/-}$ tissues than in $Arf^{-/-}$. In summary, Dmp1α stimulates the p53 pathway by direct transactivation of the *Arf* promoter in response to oncogenic stresses (4, 8, 11) and through direct physical interaction with p53 in response to dsDNA breaks (1, 7).

The h*DMP1* gene is located on chromosome 7q21, a region often deleted in human cancers, esp. in breast cancer. The h*DMP1* locus encodes at least three splicing variants, i.e. h*DMP1*α, β, and γ (2; Fig. 2). The full-length h*DMP1*α gene corresponds to the murine *Dmp1* gene that positively regulates the $p19^{Arf}$-p53 pathway. On the

other hand, the hDMP1β and γ isoforms lack the DNA-binding domain, and hDMP1β is dominant-negative for hDMP1α on myeloid differentiation and *CD13* induction. Thus, splicing alterations involving h*DMP1β/γ* may contribute to human carcinogenesis. We recently analyzed 51 primary lung cancer and 110 breast cancer samples; LOH of h*DMP1* was found in 35 % of lung cancer (11) and 42 % of breast cancer (5) cases. This finding is significant since the frequency of h*DMP1* involvement is equivalent to that of *INK4a/ARF* or *p53* in human breast cancer. LOH of h*DMP1* was found in a mutually exclusive fashion with that of *INK4a/ARF* and/or *p53* in both tumor types, suggesting that hemizygous h*DMP1* deletion defines a new disease entity with distinct prognosis. Moreover, the oncogenic splicing variant h*DMP1β* was overexpressed in up to 30 % of our breast cancer specimen. Thus, *we hypothesize that hDMP1β plays a key role in the initiation and progression of breast cancer, and thus it may be a novel prognostic factor and a new therapeutic target.*

Project 1: Dmp1-p53/YY1 interaction in lung carcinogenesis (collaboration with Dr. G. Sui). Accumulating pieces of evidence show that the nuclear matrix protein YY1 (Yin Yang 1) exhibit its oncogenic activity by activating the expression of oncogenes (e.g. *c-Myc, c-Fos, B23, Cdc6*), decreasing the levels of tumor suppressor proteins (RB, p53), and histone acetylation/methylation. YY1 binds to all of p53, Mdm2, and Arf to accelerate Mdm2-mediated polyubiquitination of p53 (Fig. 1). Of note, YY1 binds to the polycomb protein EZH2 (histone methyltransferase) to regulate gene expression through epigenetic mechanisms, without directly binding to DNA. The epigenetic regulation of eukaryotic genomes has been shown to impart the stability on DNA sequence and to contribute to the maintenance of genomic integrity. More than 80 % of human cancers overexpress YY1, and depletion of YY1 by shRNA inhibits the clonogenicity, migration, invasion, and tumorigenesis of breast cancer cells. ***We therefore hypothesize that overexpression of YY1 plays a universal role in carcinogenesis through epigenetic dysregulation of gene expression.*** To address this, we have developed *pTREtight-HAYY1* transgenic mice. If YY1 has oncogenic activities *in vivo*, we will be able to establish drug-inducible/de-inducible transgenic models for breast cancer by crossing *dox-HAYY1* mice with tissue-specific *rtTA* mice. The tumors will be analyzed for the expression of nuclear proteins described above. Chromatin immunoprecipitation will be conducted using the lysates from tumors to study the binding of YY1 to possible target genomic loci to demonstrate the mechanisms of tumorigenesis *in vivo*. For translational research, i) we will establish YY1 and its interacting partners for novel biomarker of solid tumors, and ii) we will design antisense oligonucleotides (AONs) for these molecules for future cancer therapy.

Recent studies show a novel function of YY1 in breast cancer metastasis through initiation of the epithelial-mesenchymal transition, which is associated with increased cancer stem cell activity. YY1 forms an active complex with HIF1α that accelerates tumorigenesis and metastasis to increase *VEGF* expression, stimulating systemic angiogenesis. Our study shows that 1) Dmp1α upregulates *Thbs1*, an inhibitor of angiogenesis, 2) it increases the transcription of *Egr1* that inhibits cancer metastasis. Moreover, oncogene-driven mouse models of cancer exhibit more aggressive phenotypes with invasion/metastasis in *Dmp1*-deficient mice. Since Dmp1α physically interacts with YY1, ***we hypothesize that YY1 overexpression and Dmp1 deletion will synergize in breast cancer development by increasing tumorigenicity, invasion, and angiogenesis/metastasis***. This possibility will be tested *in vivo* by crossing *dox-HAYY1;rtTA* mice with *Dmp1 K.O.* mice.

Project 2: <u>Roles of DMP1β/γ in cell cycle progression, mammary carcinogenesis, and genomic instability.</u> Emerging evidence suggests that aberrant RNA splicing contributes to essential phenotypes associated with transformed cells. We found that the h*DMP1β* protein is overexpressed in 55 % of human breast cancers (2), which overlaps with *HER2* or c-*Myc* expression, and that ectopic expression of hDMP1β stimulates cell proliferation in both normal breast epithelial and cancer cells in a p53-independent fashion (Figures). However, the molecular mechanism of cell cycle progression driven by hDMP1β/γ has not been clarified. In this study, we will study the splicing alterations of the h*DMP1* locus that result in hDMP1β/γ overexpression, demonstrate the oncogenic activity of these splice variants, and finally translate the findings to clinical levels. ***Our central hypothesis is that hDMP1β/γ promotes breast epithelial carcinogenesis by downregulating the RB protein, increasing genomic instability, affecting the stem cell activity of tumor-initiating cells. We also posit that DMP1β overexpression and Dmp1α-loss will collaborate in mammary carcinogenesis.***

In **<u>Aim 1</u>**, we will elucidate the signaling pathways that affect the *hDMP1* splicing. Currently our data show that oncogenic stress increases the *DMP1β/α* ratio, but we expect that other stress signals will also increase the ratio, which will be extensively studied in this Aim. We will also analyze the mechanisms of cell growth stimulation and carcinogenesis by hDMP1β/γ. The possibility of RB-dependence of the DMP1β activity (Fig. 2) will be pursued by cell proliferation assays in *RB*-null cells, protein-protein binding assays with DMP1β, and identification of DMP1β-binding proteins by mass spectrometric analyses followed by IP-Western blottings. We will conduct mammosphere stem cell assays using shRNAs to study how each *DMP1* splice variant affects stem cell growth of mammary epithelial cells. The **<u>Aim 2</u>** is to detect splicing alterations for h*DMP1* in human breast cancer specimens and study their prognostic values. Pairs of breast cancer cells (tumor, normal) will be obtained from the tumor bank and mRNAs and proteins will be analyzed by real-time PCR and immunohistochemistry, respectively. Our RNA-seq analysis shows that the *DMP1β* mRNA is highly expressed in ~55 % of ER(+)HER2(-) breast cancer. Since mammary tumors from *MMTV-DMP1β$_{V5His}$* mice have high cyclin D1 and Ki67 expression, we are interested in the role of DMP1β in highly proliferative/invasive breast cancer, and its impact on patients' survival. In **<u>Aim 3</u>**, we will study the biological effects of DMP1β/γ overexpression on mammary glands to examine whether ectopic expression of DMP1β/γ leads to mammary tumorigenesis in mice. **<u>Aim 3a</u>:** The *DMP1β$_{V5His}$* - transgenic mice will be crossed with *Dmp1*-null mice to study the collaborative effects of DMP1β overexpression and *Dmp1α*-loss in mammary carcinogenesis. We anticipate that mammary tumors will show more aggressive phenotypes in *Dmp1$^{-/-}$;MMTV-DMP1β$_{VH}$* mice than *Dmp1$^{+/+}$;MMTV-DMP1β$_{VH}$* mice. **<u>Aim 3b</u>:** We will create *MMTV-DMP1γ* transgenic mice (FVB) to understand the role of this splice variant in breast cancer. In **<u>Aim 4</u>**, we will identify AON sequences that specifically downregulate DMP1β/γ to cause regression of cancer. We believe that DMP1β/γ will be desirable molecular targets for future cancer therapy. This line of inquiry has direct translational importance for the disease with significant public health impact.

Project 3: <u>Roles of MEKK1 in stress signaling and tumorigenesis</u>. The serine/threonine kinase Mekk1 is activated by a variety of oxidative stress signaling including dsDNA breaks by γ-radiation (Fig. 1). Our preliminary study shows that Dmp1 (Dmp1α) is a direct target for Mekk1-mediated phosphorylation and promoter activation. ***We hypothesize that activated Mekk1 directly phosphorylates Dmp1 within the DNA-binding domain for Dmp1 to bind to DNA.*** We will

clarify the molecular mechanisms for Mekk1-mediated Dmp1 activation by pursuing the following Specific Aims. **Aim 1:** To elucidate the roles of Mekk1 on Dmp1 function. We will study both Dmp1 phosphorylation-dependent and -independent functions of Mekk1 on Dmp1 activation. We expect to show that the Mekk1-Dmp1 pathway is a gateway for dsDNA break signaling, leading to p53 activation through direct Dmp1-p53 binding (Fig. 1). **Aim 2:** To study the effect of Mekk1 on Dmp1 levels in response to stress signaling *in vivo*. Both *Mekk1*$^{+/+}$ and *Mekk1*$^{-/-}$ mice will be exposed to a variety of stress-inducing agents (e.g. whole body γ-irradiation, doxorubicin/etoposide injection) to study the level of expression of Dmp1, p53, and its target genes in tissues. **Aim 3:** To cross *Mekk1*-deficient mice with *HER2/neu* or *K-Ras*^{LA}-transgenic mice to study the role of Mekk1 in solid tumor suppression. If Mekk1 is a critical mediator in oncogenic signaling to the Dmp1-p53 pathway, loss of *Mekk1* will significantly affect oncogene-induced carcinogenesis. **Aim 4:** To elucidate the involvement of MEKK1 in human breast cancer and determine its prognostic values. Genomic DNA will be isolated from clinical specimen and LOH analysis for *ATM,* c-*ABL*, *MEKK1,* h*DMP1,* and *p53* will be conducted. We expect to show the LOH for *MEKK1* is mutually exclusive of that of c-*ABL* or h*DMP1*, and is associated shorter survival of patients. Immunohistochemical studies will also be performed for MEKK1 to demonstrate its role in breast cancer development.

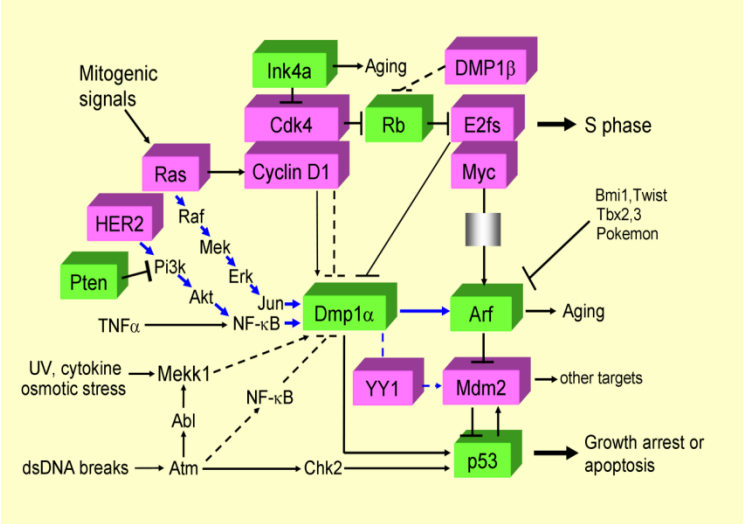

Figure 1. Oncogenic and tumor suppressive signaling pathways involving Dmp1. Arf is induced by potentially oncogenic signals stemming from overexpression of oncogenes such as c-Myc, E2F1, and activated Ras, which quenches inappropriate mitogenic signaling by diverting incipient cancer cells to undergo p53-dependent growth arrest or cell death. Dmp1α transactivates the *Arf* promoter in response to oncogenic stresses, and physically interacts with p53 to neutralize all activities of Mdm2 to activate the p53 pathway in response to DNA damage. Both *Dmp1*$^{-/-}$ and *Dmp1*$^{+/-}$ mice show hypersensitivity to develop tumors in response to carcinogen or *γ*-irradiation. D-type cyclins inhibit Dmp1's transcriptional activity in a Cdk-independent fashion lacking E2F sites; however, it cooperates with Dmp1α to activate the *Ink4a* and *Arf* promoters to eliminate incipient tumor cells. The *Dmp1* promoter is activated by the oncogenic Ras-Raf-Mek-Erk-Jun and HER2-Pi3k-Akt-NF-κB pathways, and thus Ras or HER2-driven carcinogenesis is accelerated in *Dmp1*-deficient mice. Dmp1α physically interacts with the epigenetic modifier YY1 that affects EZH2 activity. YY1 binds to Mdm2 and Dmp1α to accelerate Mdm2-mediated polyubiquitination of p53 (Project 1). The human *DMP1* locus generates three splice variants, namely *DMP1α, β*, and *γ* with antagonizing activity (Project 2; Fig. 2). The serine/threonine kinase MEKK1 is activated by a variety of oxidative stress signaling, such as dsDNA breaks, UV, cytokines, osmotic stress, and oncogenes. It is cleaved by caspase 3 following DNA-damage to generate ΔMEKK1, which increases the Dmp1α protein by phosphorylation (Project 3). *Our current model indicates that Dmp1 is a key mediator for both p53 and Rb signalings, and thus can be a target for cancer therapy*.

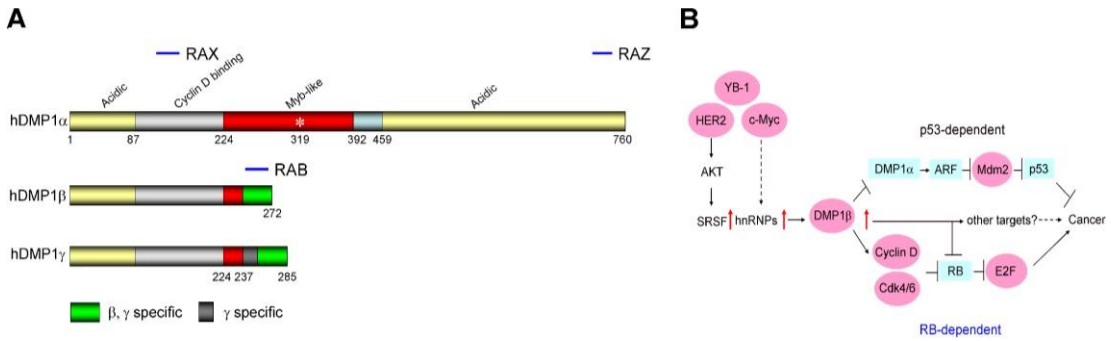

Figure 2. Splicing variants of the human *DMP1* gene and anticipated signaling pathways regulated by hDMP1β. (A) The hDMP1β and γ proteins lack most Myb-like repeats and the principal transactivation domain, and do not bind to DNA. K319 is critical for its DNA binding. **(B)** Current hypothesis. hDMP1β plays a key role in the initiation and progression of breast cancer by downregulating RB levels. Additionally, hDMP1β directly binds to hDMP1α to block its activity to induce *Arf* and activate p53. These two mechanisms will collaborate to promote carcinogenesis. *We hypothesize that increased Akt activity caused by HER2 amplification/overexpression or high c-Myc expression changes the levels of splicing factors, which will lead to altered DMP1β/α ratio and inactivation of both RB and p53 pathways.* We will analyze the mechanisms of aberrant splicing at the *hDMP1* locus in Project 2.

Publications

Primary articles:

1. Kendig RD, Kai F, Fry EA, and Inoue K*. Stabilization of the p53-DNA complex by the nuclear protein Dmp1α. Cancer Invest May 28;35(5): 301-312. doi: 10.1080/07357907.2017.1303505. Epub 2017 Apr 13.

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