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| <b>14. ABSTRACT</b><br>Heterotopic bone can form in muscle or other soft tissue that is adjacent to a fracture, blast wound, or amputation injury. This condition causes severe pain and disability. Chemokine-directed stem cell homing is an important early stage of heterotopic bone formation. CXCR4 is the most important chemokine in this procedure. Noninvasive evaluation of CXCR4 status is important for early diagnosis and treatment. We have synthesized peptide-based CXCR4 imaging agents and labeled them with near-infrared dye for in vitro and in vivo studies. Cell studies have demonstrated that these agents bind to CXCR4+ cells. Whole-body near-infrared optical images have shown high signal intensity from CXCR4+ disease sites and the liver region. Noninvasive target-specific molecular imaging has been achieved in both in vitro and in vivo studies. These imaging agents could be helpful in developing therapeutic agents, choosing receptor-positive diseases for therapy, and estimating the biological effects of therapy. |                         |                                 |                                             |                                                      |                                                   |
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## Introduction

Heterotopic bone can form in muscle or other soft tissue that is adjacent to a fracture, a blast wound, or an amputation injury and can cause severe pain and disability. Initial diagnoses are typically based on the findings of computed tomography (CT) and radiographic imaging, but these imaging methods are useful only at the later stages of the disease, when substantial and progressive bone formation is already evident. Because of the lengthy duration and progressive nature of the disease, multiple surgical procedures for resection of heterotopic bone are often necessary when the disease reaches its late stages, particularly when patients have undergone surgical amputation after blast injuries. A particularly frustrating problem for these patients is the progressive growth of bone within the amputation stump; this condition makes wearing a prosthesis difficult or impossible.

This research proposal is aimed at using molecular imaging techniques to develop new, early diagnostic and therapeutic procedures for heterotopic bone formation. We have combined the use of nuclear and near-infrared (NIR) probes with a preclinical animal model so that we can detect early disease processes by using diagnostic molecular imaging. We believe that this combination of a preclinical model and molecular imaging will lead not only to an improved understanding of the early biological processes associated with the disease but also to a method that, through imaging, can diagnose the presence of those early processes and detect their therapeutic interruption.

Microarray analysis has shown that stem cell homing is one of the important early stages of heterotopic bone formation. The release of chemokines plays an important role by leading the stem cell migration and homing to the trauma site. The most important chemokine ligand-receptor interaction is that between chemokine (C-X-C) motif ligand 12 (CXCL12; also called stromal cell-derived factor-1, SDF-1) and its exclusive receptor, chemokine (C-X-C) receptor 4 (CXCR4). CXCR4 is involved in cell migration and microenvironment interactions (1,2). Because CXCR4 is believed to play a role in the development of heterotopic bone, it may be a crucial therapeutic target, and several CXCR4 antagonists have been proposed as potential therapeutic agents. Establishing a noninvasive method for evaluating CXCR4 expression and its changes during treatment is crucial if we are to develop target-specific therapeutic agents, to select target-positive diseases, and to determine the biological effects of treatment.

We have synthesized peptide-based CXCR4 imaging agents and labeled them with NIR dye for in vitro and in vivo studies. Cell studies have demonstrated that these agents bind to CXCR4<sup>+</sup> cells. Whole-body NIR optical images show high signal intensity at the CXCR4<sup>+</sup> disease site and in the liver region. In addition, <sup>18</sup>F-fluoro-deoxy-glucose positron emission tomography (<sup>18</sup>F-FDG PET) demonstrates the high metabolic status of CXCR4<sup>+</sup> tumors. CT images show hypervascularity in the same region. We have achieved noninvasive, target-specific molecular imaging in both in vitro and in vivo studies. These techniques could be helpful in developing therapeutic agents, choosing receptor-positive diseases for therapy, and estimating the biological effects of therapy.

## Body

Heterotopic bone can form in muscle or other soft tissue that is adjacent to a fracture, a blast wound, or an amputation injury and can cause severe pain and disability. Initial diagnoses are typically based on the findings of computed tomography (CT) and radiographic imaging, but these imaging methods are useful only at the later stages of the disease, when substantial and progressive bone formation is already evident. Because of the lengthy duration and progressive nature of the disease, multiple surgical procedures for resection of heterotopic bone are often necessary when the disease reaches its late stages, particularly when patients have undergone surgical amputation after blast injuries. A particularly frustrating problem for these patients is the progressive growth of bone within the amputation stump; this condition makes wearing a prosthesis difficult or impossible.

This research proposal is aimed at using molecular imaging techniques to develop new, early diagnostic and therapeutic procedures for heterotopic bone formation. We have combined the use of nuclear and near-infrared (NIR) probes with a preclinical animal model so that we can detect early disease processes by using diagnostic molecular imaging. We believe that this combination of a preclinical model and molecular imaging will lead not only to an improved understanding of the early biological processes associated with the disease but also to a method that, through imaging, can diagnose the presence of those early processes and detect their therapeutic interruption.

Microarray analysis has shown that stem cell homing is one of the important early stages of heterotopic bone formation. The release of chemokines plays an important role by leading the stem cell migration and homing to the trauma site. Chemokines are small, secreted proteins that form what is currently the largest known family of cytokines. They mediate their effects through a family of receptors coupled to G proteins, and they act as chemoattractants and as activators of specific types of leucocytes in a variety of immune, inflammatory, and malignant diseases (3). The most important chemokine ligand-receptor interaction is that between chemokine (C-X-C) motif ligand 12 (CXCL12; also called stromal cell-derived factor-1, SDF-1) and its exclusive receptor, chemokine (C-X-C) receptor 4 (CXCR4). The interaction between CXCL12 and CXCR4 may have a distinctive biological role. In fact, the CXCL12-CXCR4 axis may be the key to organ-specific metastasis and the promotion of cell migration, with resulting implications for therapy (4).

CXCR4 is involved in inflammatory diseases (5), fibrosis (6), cancer (7,8), physiological cell migration, and pathological processes such as metastasis (9) and heterotopic bone formation (10-12). Because CXCR4 plays such an important role in all of these diseases, it may be a crucial therapeutic target, and several CXCR4 antagonists have been proposed as potential therapeutic agents (6). Therefore, establishing a noninvasive method for evaluating CXCR4 status is vital if we are to develop new drugs, estimate their efficacy, and properly select patients for treatment.

In attempting to establish a method for detecting CXCR4, one group of researchers successfully labeled the peptide with indium-111. They then performed a biodistribution

study of this labeled agent and found that, 6 hours after injection, the tumor-to-muscle distribution ratio of the agent was 4.43 (13). Another group labeled CXCR4 with fluorophore and demonstrated in vitro cell binding (14). However, no in vivo imaging results were published.

The purpose of our study is to develop CXCR4 agents that can be visualized by imaging so that we can increase our understanding of the action of CXCR4 both in vitro and in vivo. We have synthesized CXCR4 agents, labeled them with reporters, and tested the binding of these synthetic agents to human osteosarcoma cells that express CXCR4 (15) and form heterotopic bone. We have used molecular imaging to determine the whole-body distribution of these agents. Our cell binding studies have demonstrated that these agents bind to human tumor cells. Optical whole-body images show that CXCR4 exhibits high signal intensity in tumors and in the liver region. In addition,  $^{18}\text{F}$ -fluoro-deoxy-glucose positron emission tomography ( $^{18}\text{F}$ -FDG PET) shows the high metabolic status of CXCR4<sup>+</sup> tumors. CT images acquired after the injection of a contrast agent into the tumor vasculature demonstrate high signal intensity. Our osteosarcoma model exhibits a high level of x-ray signals.

In conclusion, we have demonstrated that synthetic CXCR4 agents can be visualized by imaging, both in vitro and in vivo. These labeled CXCR4 agents may be useful for evaluating CXCR4 chemokine receptor status in preclinical research. Noninvasive molecular imaging methods can be used to determine the distribution of these CXCR4 agents in various tissues, organs, and disease models. We can also measure the uptake of these agents and correlate that uptake with the disease response. Because these CXCR4 agents can be imaged, we will be able to use a biological dose to further develop image-guided therapy. We can use imaging methods to select target-positive diseases, define the dosage and frequency of administration, and follow the agents' passage through the body. These agents may enable us to detect CXCR4 receptor-positive tumors or diseases.

## **Key Research Accomplishments**

### **Synthesis of CXCR4 compounds**

We have generated three forms of CXCR4 imaging agents for this study. The NIR agent is used for optical imaging, whereas the dual-labeled NIR/single photon emission computed tomography (SPECT) and dual-labeled NIR/PET agents can be used for optical and nuclear imaging. The NIR agent, which is stable and has a long shelf life, provides a convenient means of testing the relationship between structure and binding affinity. We can also use this agent to screen the target expression level in vitro and in vivo. The chemical design of the dual-labeled agents allows us to easily exchange the optical and nuclear components while maintaining the same target specificity. The optical component can be replaced by chemotherapy agents, and the diagnostic isotope can be replaced by therapeutic isotopes. This exchange of components allows us to use the same target components to create “seek, treat, and see” or target-specific chemoradiotherapeutic agents. We have also synthesized several agents with different structures and binding properties.

### **In vitro and in vivo models**

The human osteosarcoma cell line Saos-2 was purchased from the American Type Culture Collection (ATCC; Manassas, VA). This cell line consistently expresses a high level of CXCR4 (2) during proliferation and growth of the tumor. Thus, the cell line provides a wide window for evaluating our agents both in vitro and in vivo.

### **Fluorescent confocal microscope imaging**

We used a fluorescent confocal microscope to confirm and compare the binding of our agents to CXCR4<sup>+</sup> cells. Cells were harvested from culture, an NIR-labeled CXCR4 agent was added, and the mixture was incubated for 60 min at 37°C. Cells were washed in phosphate-buffered saline (PBS). Sytox green (Molecular Probes; Invitrogen, Carlsbad, CA) in 95% ethanol was added, and cells were incubated for 15 min at 4°C to fix the cells and stain the cell nuclei. Stained cells were then transferred to a slide and mounted for microscopic examination. Images were recorded by a FluoView 1000 confocal microscope (Olympus America, Center Valley, PA). The microscope was equipped with an excitation (ex) light source and emission (em) filters so that it could detect and distinguish signals from CXCR4 agent or NIR dye (ex/em, 765/810 nm) and from cell nuclei (ex/em, 488/510 nm). In the microscopic images, signal intensities were recorded from one slice of cell z-stacks. Sytox green and CXCR4 agent were pseudocolored green (em, 510 nm), and NIR dye signals were pseudocolored red (em, 810 nm).

### **In vivo imaging**

We have successfully established a Saos-2 xenograft model for studying our agents in vivo. Tumors were induced in animals by subcutaneous injection of tumor cells. NIR-labeled CXCR4 (10 nmol) was injected into the tumor-bearing mice through the tail vein. The animals were imaged up to 3 days after the injection with a KODAK In-Vivo Multispectral System FX (Carestream Health Molecular Imaging, New Haven, CT). For the purpose of comparison, all images were captured over time according to the same standard protocol and were saved as uncompressed 16-bit tagged image format (TIF) files for further analysis. Ominipaque (GE Healthcare, Princeton, NJ) was injected for determination of tumor vascularity; images were acquired by CT (microCAT II, Siemens Medical Solutions, Hoffman Estates, IL). The metabolic status of tumors was determined by PET (Inveon; Siemens Medical Solutions, Malvern, PA) after injection of <sup>18</sup>F-FDG (Cyclotope, Houston, TX). All images were acquired with the manufacturers' software. ImageJ (National Institutes of Health, Bethesda, MD) and Amira (Visage Imaging, San Diego, CA) were used for 3D reconstructions, volume rendering, and signal-to-background ratio analysis.

### **Pathology**

To validate our whole-body imaging results at the pathological level, we dissected the animals and took samples of organs exhibiting high signal intensity (thymus, liver, and tumor) and of muscle (as a negative control) for hematoxylin and eosin (H&E) staining. The pathology images confirmed the distribution of the labeled agents in these tissues.

## Reportable Outcomes

### Cell binding study

A side-by-side comparison study showed that the NIR CXCR4 agent bound to osteosarcoma cells, but the free NIR dye did not bind to most cells (Fig. 1, A and E). Merging the NIR images, the cell nuclei staining images, and the differential interference contrast (DIC) images verified the origin of the NIR signals (Fig. 1, B and F). No NIR signal was expressed by most of the cells incubated with the free NIR dye (Fig. 1B). In contrast, all cells incubated with the CXCR4 agent expressed strong NIR signals. Furthermore, those NIR signals were not colocated with the cell nuclei (Fig. 1F). Direct comparison of the NIR signal intensity of free dye and CXCR4 at the level of a single cell (Fig. 1, C and G) showed that there was no detectable NIR signal in the cell labeled with free dye (Fig. 1C). The uneven intensity of the NIR signal in the cell bound to the CXCR4 agent (Fig. 1G) suggests that this agent may bind to specific cellular compartments. Quantitative signal intensity comparisons (Fig. 1, D and H) showed that the signal intensity of the free NIR dye was at a background level, whereas the signal intensity of CXCR4 differed at various locations within the cell.

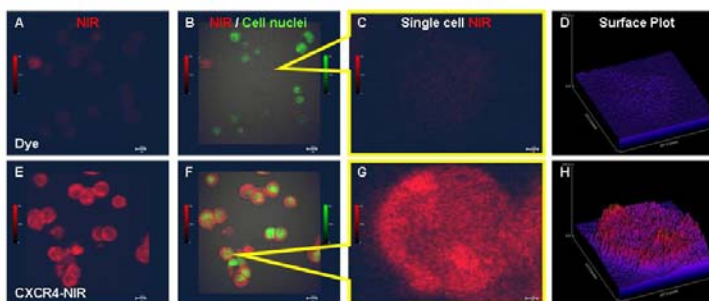


Figure 1. Confocal images demonstrating uptake of a CXCR4 agent in human osteosarcoma cells. A. Cells incubated with free near-infrared (NIR) dye. B. Merged image of NIR uptake, cell nuclei, and bright field showing cell morphology, NIR signal intensity, and location within nuclei. C. High-magnification image of free dye uptake by a single cell. D. Plot of free dye signal intensity. E. CXCR4 agent binding to osteosarcoma cell. F. Merged image of CXCR4 agent, cell nuclei, and bright field showing cell morphology, CXCR4 signal intensity, and location within nuclei. G. High-magnification image of a single cell binding to CXCR4. H. Plot of CXCR4 signal intensity from a single cell.

### In vivo imaging

The CXCR4 agent allowed us to detect an increase in signal intensity in the osteosarcoma model as early as 7 days after inoculation of the cell. Figure 2 shows

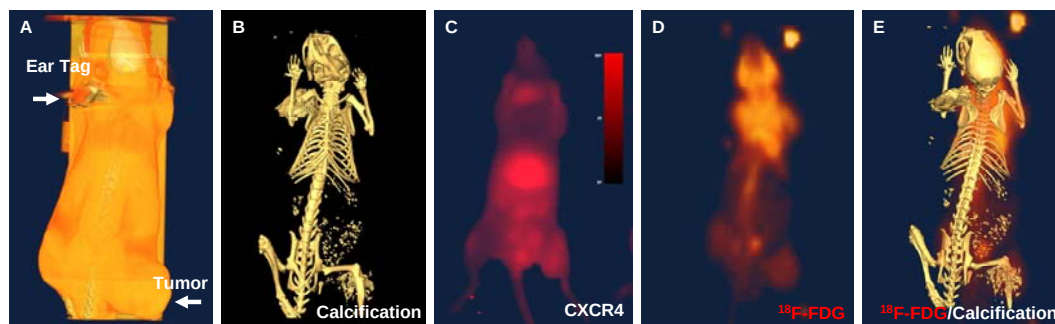


Figure 2. In vivo images of disease status in osteosarcoma xenografts. A. Whole-body CT image showing the location of the tumor. B. CT skeletal image demonstrating calcification in the tumor region. C. Near-infrared (NIR) image showing the distribution of CXCR4 agent in the thymus, liver, and osteosarcoma. D. FDG-PET image showing high level of glucose metabolism in the osteosarcoma region. E. Merged CT and PET images showing anatomical location of the tumor and whole-body CXCR4 signal distribution.

images of late-stage tumors. Whole-body CT imaging demonstrated the size and location of the tumors (Fig. 2A). Images of the skeleton demonstrated calcification in osteosarcoma tumors (Fig. 2B). NIR images showed that the CXCR4 agent was localized to the thymus, the liver, and the tumor (Fig. 2C). Tumor-to-background ratios ranged from 1.01 to 1.75 during a 48-hour period (n=8). In addition,  $^{18}\text{F}$ -FDG PET imaging demonstrated the high level of glucose metabolism exhibited by this osteosarcoma (Fig. 1D). The merged skeletal and PET images demonstrated the location of the tumor, the calcification in the tumor region, and the level of glucose metabolism in the tumor (Fig. 2E).

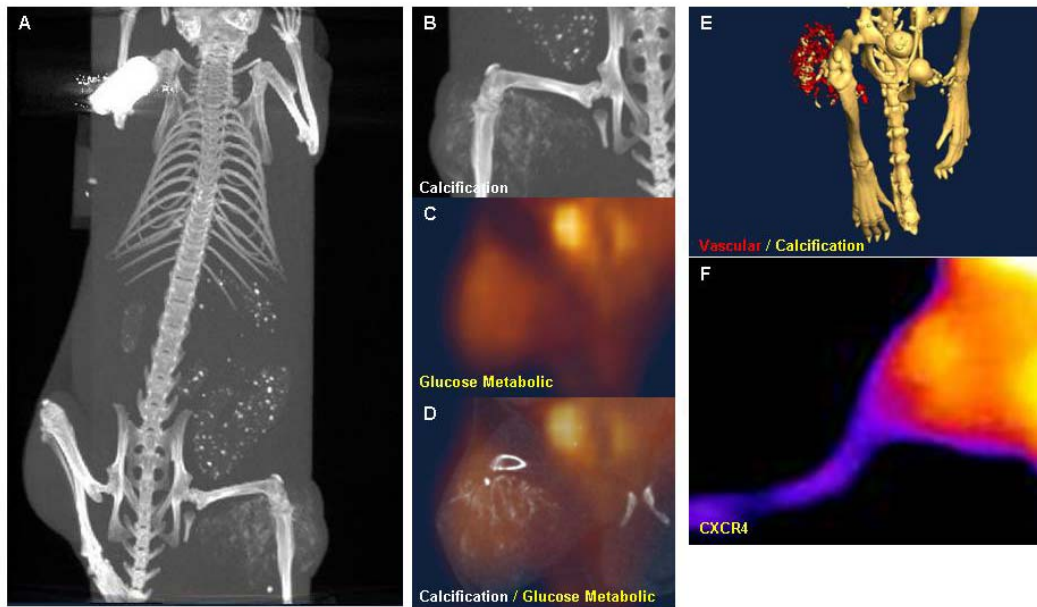


Figure 3. High magnification of in vivo images of osteosarcoma disease status. A. Whole-body CT image showing the tumor location with calcification. B. CT image demonstrating calcification in the tumor region. C. FDG-PET image showing a high level of glucose metabolism in the same region. D. Merged CT and PET images of B and C. E. Merged CT skeletal image and vasculature contrast images showing hypervascularity and calcification in the tumor region. F. Optical image showing strong CXCR4 signal in the tumor region.

Figure 3 shows a close-up imaging analysis of osteosarcoma disease status. The whole-body image (Fig. 3A) shows the location of the tumor, and a CT image (Fig. 3B) details the calcification of the osteosarcoma. The bony component of the tumor has invaded beyond the tumor mass. The center of the tumor exhibits a high level of glucose uptake (Fig. 3C). Merged  $^{18}\text{F}$ -FDG PET and CT images (Fig. 3D) demonstrate the relationship between glucose uptake and tumor mass. Merged vasculature contrast and skeletal images (Fig. 3E) show the hypervascularity of the tumor, and optical images (Fig. 3F) demonstrate the strength of the CXCR4 signal in the tumor.

### Pathological analysis

To validate our whole-body imaging results at the pathological level, we dissected the animals and took samples from organs exhibiting high signal intensity (thymus, liver, and tumor) and from muscle tissue (as a negative control) for H&E staining. The pathology images confirmed that the tissues with the highest signal intensity were from the thymus, liver, and tumor (Fig. 4).

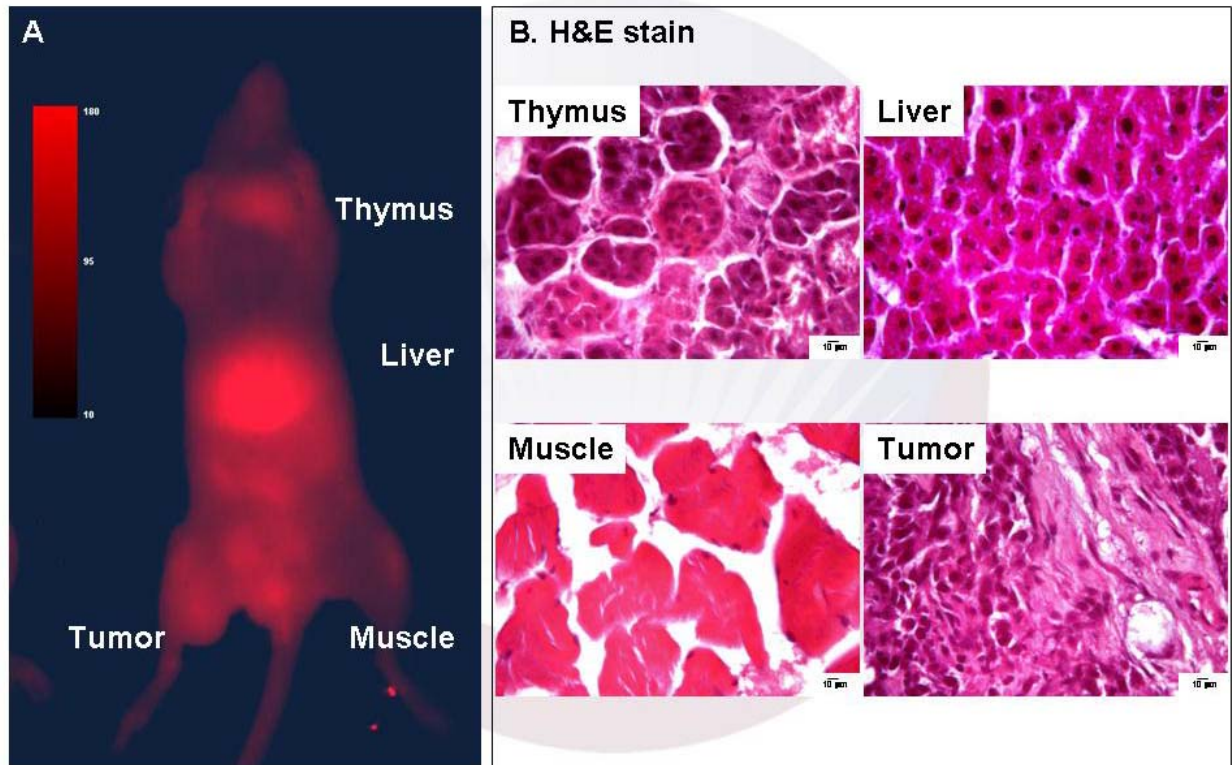


Figure 4. Pathology confirmed that the tissues exhibiting the highest signal intensity were from the thymus, liver, and tumor.

### Conclusion

CXCR4, the most important chemokine receptor, plays a crucial role in disease progression and in nonphysiological cell migration, as is found in heterotopic bone formation. The action of CXCR4 in many pathological processes makes it an important therapeutic target. Several CXCR4 antagonists have been developed and are currently undergoing clinical trials. A noninvasive method for evaluating and monitoring biological changes in CXCR4 status is crucial for both preclinical studies and ongoing clinical trials.

We have generated CXCR4 agents labeled with NIR, NIR/SPECT, and NIR/PET. In this report we have described an optical imaging agent for CXCR4 studies. Our studies have demonstrated that this agent can be used for both in vitro and in vivo studies. The results of the cell binding studies demonstrate that the target component of this agent is important, as shown by the negative binding results of studies using NIR dye alone. It is important to validate the origin of the signal and its relationship to a specific cell compartment by using confocal microscopy and collecting more than one channel signal from a single cell slice. These results represent the true data despite the low signal

intensity caused by limited photo counts. In vivo imaging demonstrates this agent-specific binding to CXCR4<sup>+</sup> organs and tissue. Pathology results validate the entire body of the data.

The chemistry design of the dual-labeled agents will allow us to easily exchange the optical and nuclear components while maintaining the same target specificity. The exchangeable components will make it possible for us to develop “seek, treat and see” target-specific agents in the future.

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