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Small molecule regulation of nuclear receptors

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Small molecule regulation of nuclear receptors

by

Neal David Freedman

DISSERTATION

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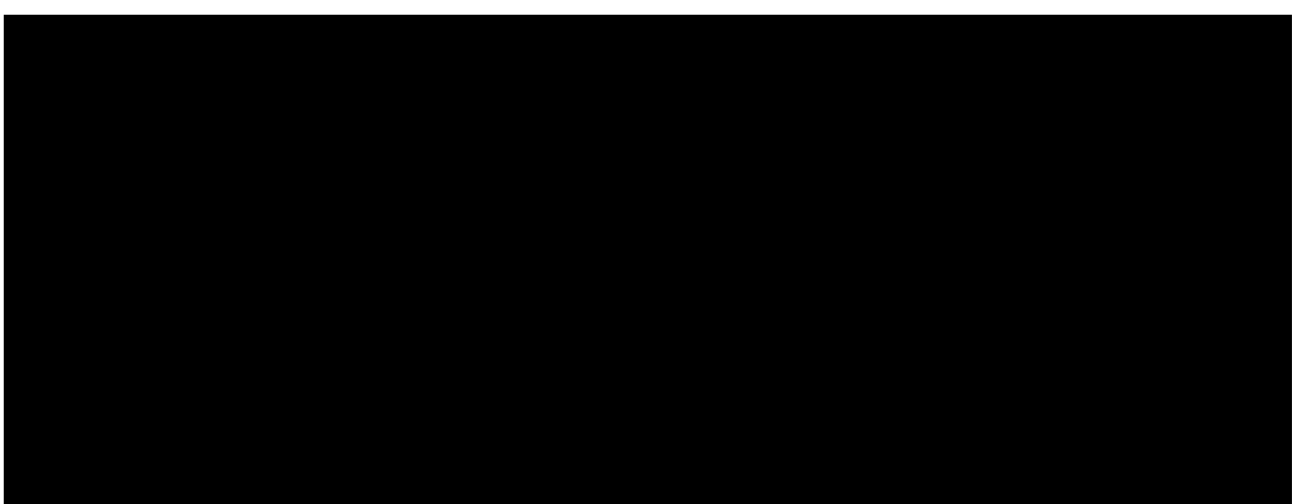

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I dedicate this work to

Mara Freedman
Ira Herskowitz
Bernard Witkin

Their wisdom, humor, zest for life, and untimely passing have affected me deeply and framed my goals and aspirations.

I will remember them always.

Acknowledgements:

In many ways, UCSF is the ideal environment for graduate education. While postdoctoral fellows may say UCSF grad-students are spoiled, I say they are jealous of the phenomenal training, support, and lifestyle we enjoy. UCSF was the first place I have ever been where student learning comes first. I have relished learning in such an environment. To all my professors and classmates—thank you.

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The other members of the Yamamoto lab have also helped me tremendously. I feel very lucky to have had the privilege of interacting with my brilliant and warm Yamamoto lab colleagues on a daily basis. I will miss everyone. I am particularly indebted to Val.

Val s ability to multi-task: to do twelve things at once with a smile on her face, has never ceased to amaze me. I cannot adequately thank her for all of her efforts on my behalf. Thanks also to Sol for helping me pour plate after plate after plate for the worm project, again always with a smile. I would have been in graduate school another year, at least, if it were not for Sol. I would also like to thank everyone in the Yamamoto Lab for their help and support, particularly Wally for getting me started and helping perform many of the experiments detailed in Chapter 2, Inez and Hans for reading everything I wrote without fail, Sebastiaan for performing the transfection experiment with N525 in mammalian cells (Chapter 1, Figure 4), Marc VG for many interesting discussions, some about science and experimental help, and Kol for running all of the qRT-PCR asssays (Chapter 2: Figure 8). I also would like to thank my collaborators: Coleen Murphy and the Kenyon lab for all of their help with the microarrays and lifespan experiments; and Adam Antebi for allowing me to work in his lab and for strains, reagents, and many interesting discussions.

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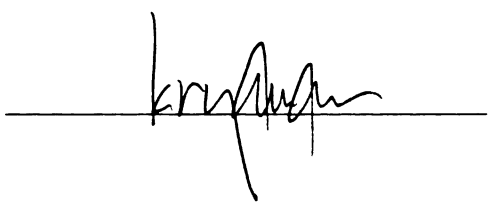
Abstract:

Neal David Freedman

Small molecules, such as steroid hormones, are key regulators of intracellular communication. Steroid hormones exert their action by binding to nuclear receptors in target organs, allowing nuclear receptors to translate endocrine signals into physiological outputs by regulating the transcription of target genes. My thesis has focused on two poorly understood aspects of physiological regulation by small molecules. In Chapter 1, I investigated the mechanism by which cholesterol-derived steroid hormones control nuclear receptor function in mammalian cells, focusing on the regulation of the glucocorticoid receptor (GR) intracellular localization by the steroid hormone corticosterone. Binding corticosterone changes the localization of GR from the cytoplasm to the nucleus. Using the genetics of *S. cerevisiae*, I identified an import receptor, Sxm1, critical for GR nuclear import in yeast. Two metazoan homologues of Sxm1, importin 7 and importin 8, can bind GR, and importin 7 can import a fragment of GR in an *in vitro* nuclear import assay. Interestingly, importin 7 and importin 8 bound GR even in the absence of hormone, suggesting that hormonal control of localization lies downstream of import receptor binding.

In Chapter 2, I used *C. elegans* to probe the roles of small molecules in regulating physiogical processes. In the absence of cholesterol, worms display defects in growth, reproduction, molting, dauer formation, and gonandal migration (mig). As cholesterol serves as a precursor for a subset of nuclear receptor ligands in mammalian cells, I tested in worms the effects of inhibitors of mammalian cholesterol metabolism. Worms grown in the presence of ketoconazole displayed growth, mig, and reproductive defects. I determined that the mig but not the growth and reproductive phenotypes required the function of a nuclear receptor, *daf-12*, suggesting that ketoconazole may block the

formation of a cholesterol-derived ligand for *daf-12*. These data suggest that ketoconazole affects multiple *C. elegans* signaling pathways and will serve as a valuable tool for investigating the molecular role of cholesterol in *C. elegans* physiology. Lessons learned from these experiments will also likely be relevant to more complex metazoans, such as mammals.



Keith Yamamoto

Thesis Committee Chair

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Introduction:

Organisms must sense and respond to their environment, maintaining homeostasis in spite of changing external conditions. Single cells can directly respond to changes in osmolarity, nutrients, temperature, and toxins; for example, altering gene expression in times of scarcity to reduce energy expenditure. Similarly, multi-cellular organisms must respond to changing environmental conditions, however they also must coordinate the efforts of individual tissues and organs in order to produce a physiologically rational response. Thus, inter-organ communication is a key feature of multi-cellular organisms.

Hormones are endocrine messengers

Hormones can be thought of as messengers, released from neuro-endocrine glands and conveying information to spatially isolated organs (Tomkins, 1975). They were originally identified by biological activity, for example, as components of thyroid extracts that could regulate the metamorphosis of tadpoles into frogs (Dodd and Dodd, 1976). Steroid hormones were also identified by their biological function. The adrenal hormones, including aldosterone and cortisol, regulate glucose metabolism, water and salt homeostasis, and the immune system. The sex steroids: progesterone, testosterone, and estrogen regulate development, reproduction, and secondary sex characteristics. Finally, vitamin D regulates bone development and calcium metabolism (Evans, 1988). These functional studies demonstrated that steroid and thyroid hormones can regulate diverse processes in multiple organs, and both constant and discrete temporal events, from maintaining homeostasis to regulating metamorphosis. Pioneering work by Kendall and others identified the structure of steroid hormones and determined that they were derived from cholesterol (Gaunt, 1975). While such molecules are relatively simple for the cell to construct, they also lack the complexity that might be expected of molecules that have such disperate effects in different organs. However, the discovery that hormones bind protein "receptors" in the tissues of target organs, and subsequent investigations into the

biology of hormone receptors has yielded insight into this apparent paradox (Yamamoto, 1985).

Steroid hormone receptors are molecular switches, regulated by hormone.

Steroid and thyroid hormone receptors were first identified by their ability to bind radioactively labeled hormones (Tomkins and Martin, 1970). The subsequent molecular cloning of the glucocorticoid receptor (which binds the adrenal steroid cortisol) and the receptors for thyroid hormone and the sex steroids, identified hormone receptors as a family of transcriptional regulatory factors. These proteins have conserved domains for hormone (ligand) binding (LBD), DNA binding (DBD), nuclear localization (NL), transcriptional activation, and dimerization among others (Evans, 1988; Baumann *et al.*, 1999). The cloning of the steroid receptor genes also allowed the identification of additional proteins with these conserved domains, including regulators of fatty acid and glucose metabolism (Michalik *et al.*, 2003), cholesterol and bile acids (Lu *et al.*, 2001; Makishima *et al.*, 2002), and xenobiotics (Xie and Evans, 2001). Together, this protein family was termed the nuclear receptors (NR) (Mangelsdorf *et al.*, 1995).

Steroid hormones act as molecular switches, transforming steroid receptors from a functionally inactive form to a form that regulates transcription, in a dose-dependent manner (Picard *et al.*, 1988). Hormone binding signals steroid receptors to enter the nucleus and bind to discrete nuclear sites, altering transcriptional initiation at nearby promoters (Yamamoto, 1985; Picard and Yamamoto, 1987). Although hormone is a fundamental regulator of steroid receptor function, the mechanism of this transformation remains poorly understood. In Chapter 1, I discuss investigations into the mechanism by which glucocorticoids regulate the localization of one steroid hormone receptor, the glucocorticoid receptor (GR). GR is an important transcriptional regulator with roles in energy metabolism, respiration, the stress response, and the immune system.

Hormone has striking control over GR localization: in the absence of hormone, GR is almost completely localized to the cytoplasm; upon hormone binding, GR redistributes to the nucleus within minutes of hormone addition. Studies of GR localization will yield insights into how hormone regulates GR function and how cells regulate nuclear-cytoplasmic transport. Furthermore, as glucocorticoids are often prescribed clinically, understanding how hormone controls GR localization may allow the development of novel drugs that can modulate GR function.

I hypothesized that in the absence of hormone, GR fails to bind to nuclear import receptors, a family of proteins that transport large proteins into the nucleus. Hormone binding would then cause a conformational change in GR allowing it to bind nuclear import machinery and enter the nucleus. As described in Chapter 1 of this thesis, I developed a yeast assay to identify nuclear import receptors that transport GR into the nucleus and tested whether hormone regulates the interaction of GR with these import receptors.

How do organisms use hormones to regulate physiologal networks?

A major challenge remains to understand how the transcriptional regulatory programs initiated by NRs are controlled in complex physiological settings. For example, consider the transition from tadpole to frog. What are the signaling pathways responsible for releasing thyroid hormone at morphogenesis? How do frogs use transcriptional regulatory mechanisms to ensure that TR regulates the proper downstream targets at the correct time? How are these genes similar and different from those regulated by TR in an adult frog? Current models suggest that NRs integrate multiple types of information, such as the hormone, DNA sequence or structure, and cofactor interactions to select which genes to regulate. NRs bind response elements as subunits of gene specific transcriptional regulatory complexes. Together, the composition and interactions of these subunits

specify high or low gene expression (Yamamoto *et al.*, 1998). For example at composite NR binding sites, NRs can act either as transcriptional activators or repressors, based on the composition of signal regulated transcription factors bound to adjacent DNA sequence (Lefstin and Yamamoto, 1998). How do organisms coordinate these regulatory mechanisms to ensure proper gene expression?

Research into such topics has been difficult in mammalian systems, due to the long generation times of mammals and the difficulty performing forward genetics. However, the nematode *C. elegans* has many advantages for these studies. *C. elegans* is small (1.5 mm adult), has a 3-day life cycle, is easily propagated in the laboratory, a regular and fully mapped cell lineage, and with 959 somatic cells, a relatively simple anatomy (Riddle *et al.*, 1997). It has excellent forward genetics and with the advent of RNAi technology (Fire *et al.*, 1998), excellent reverse genetics. The sequencing of the *C. elegans* genome has led to the development of genomic tools, including microarray analysis (Reinke *et al.*, 2000; Murphy *et al.*, 2003), RNAi libraries of expressed genes (Kamath and Ahringer, 2003; Kamath *et al.*, 2003), expression libraries of *C. elegans* open reading frames (Reboul *et al.*, 2003), and comprehensive 2-hybrid protein interaction maps (Li *et al.*, 2004). Furthermore, *C. elegans* has 284 NRs (Van Gilst *et al.*, 2002; Gissendanner *et al.*, 2004), several of which have been identified in genetic screens as critical regulators of worm biology. Thus, studying NRs in worms has the potential to yield considerable insights into the logic organisms use to control nuclear receptor signal transduction.

Cholesterol, the starting point of steroid hormone synthesis in mammalian cells, is required for worm development and reproduction. Worms arrest at various stages during development when grown in the absence of cholesterol, however the reasons for this arrest are not understood. One possibility is that cholesterol metabolites are used as signaling molecules in *C. elegans;* some of these molecules may serve as ligands for

NRs. As described in Chapter 2 of this thesis, we treated *C. elegans* with pharmacological inhibitors of mammalian steroid biosynthesis, and looked for worms with aberrant phenotypes. These experiments have the potential to identify pathways that control the synthesis and degradation of cholesterol metabolites, delineate signaling pathways that use cholesterol metabolites, identify metabolites that serve as nuclear receptor ligands, and yield insights into how organisms regulate cholesterol metabolism to specifically alter gene expression in a physiological context.

Chapter 1: Identifying nuclear transport receptors for the glucocorticoid receptor.

Chapter 1: Abstract

The vertebrate glucocorticoid receptor (GR) is cytoplasmic without hormone, localizes to the nucleus after hormone binding, and redistributes to the cytoplasm during hormone withdrawal. GR has two nuclear localization signals (NLS): NL1 is similar in sequence to the SV40 NLS; NL2 is poorly defined, residing in the ligand binding domain. I found that GR displayed similar hormone-regulated compartmentalization in *Saccharomyces cerevisiae*, and required the Sxm1 nuclear import receptor for NL2 mediated import and the MSN5 nuclear export receptor for redistribution to the cytoplasm. Two metazoan homologues of Sxm1, importin 7 and importin 8, bound both NL1 and NL2, whereas importin α selectively bound NL1. In an *in vitro* nuclear import assay, both importin 7 and the importin α-importin β heterodimer could import a GR NL1 fragment. Under these conditions, full-length GR localized to nuclei in the presence but not absence of an unidentified component in cell extracts. Interestingly, importin 7, importin 8, and importin α bound GR even in the absence of hormone; thus, hormonal control of localization is exerted at a step downstream of import receptor binding.

Chapter 1: Introduction

In order to survive in a complex environment, cells sense external cues and commonly respond by altering gene expression, in part by regulating the intracellular localization and activity of transcriptional regulatory factors. For example, the intracellular localization of the yeast Pho4 and mammalian SREBP factors is altered in response to changes in external phosphate and circulating sterol concentrations, respectively (Kaffman *et al.*, 1998b; Nagoshi *et al.*, 1999). Similarly, changes in blood glucose levels and a wide range of physiologic stress signals modulate the circulating level of glucocorticoids, changing the activity and localization of the glucocorticoid receptor (GR). Within minutes of glucocorticoid binding, GR enters the nucleus and activates or represses target gene transcription (Nagoshi *et al.*, 1999). However, hormone binding is reversible; after hormone withdrawal, GR relocates back to the cytoplasm ($t_{1/2}$ = 10 hours) (Hache *et al.*, 1999).

Proteins and RNAs enter and exit the nucleus through the nuclear pore, a 125 MDa complex in the nuclear envelope (Stoffler *et al.*, 1999). Small molecules readily diffuse through the nuclear pore whereas, molecules greater than ~40 KDa require import and export machinery for transport (Davis, 1995; Pante and Aebi, 1995). The first nuclear localization sequence (NLS) was identified in the SV40 large T-antigen (Kalderon *et al.*, 1984). Development of an *in vitro* nuclear import assay facilitated the identification of two proteins that import substrates containing SV40 NLS-like domains. The adapter molecule importin α binds to the NLS of the substrate and the importin β transport receptor, creating a trimeric complex that imports the substrate into the nucleus through the nuclear pore (Strom and Weis, 2001).

Based on similarity to importin β, a family of nuclear import and export receptors was identified, and shown to transport a wide variety of proteins and RNAs (Gorlich

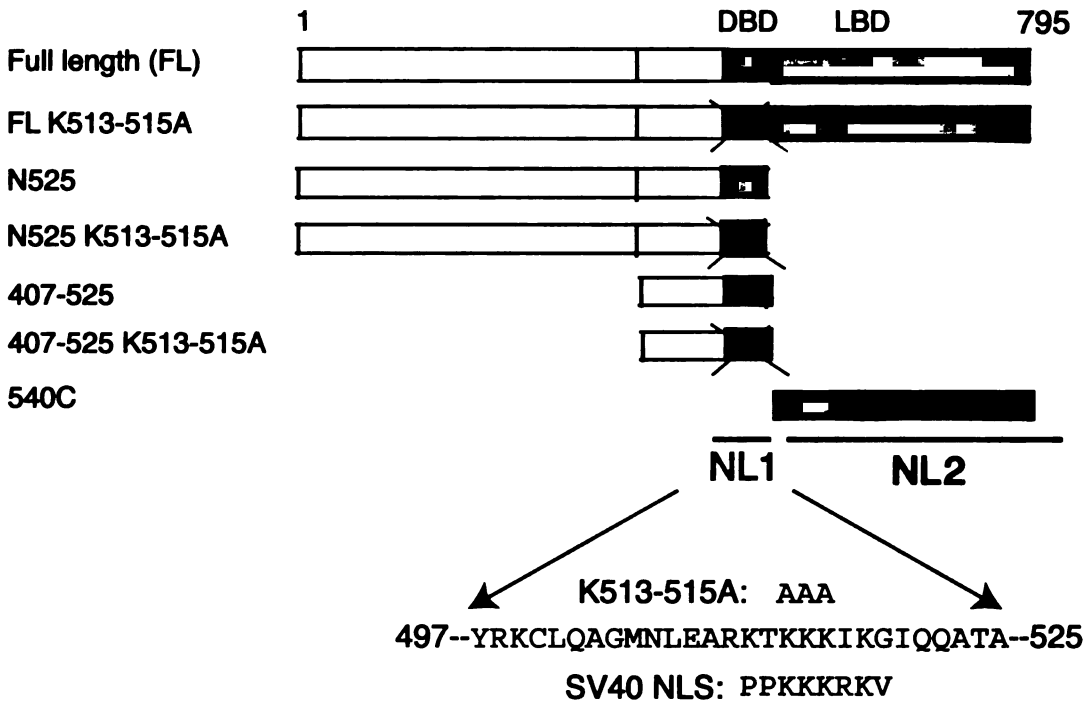


Figure 1: Constructs used in this work. Diagram shows full-length GR with positions of the DNA binding domain (DBD), ligand binding domain (LBD), NL1, and NL2, other GR constructs used, and sequence similarities between GR and the SV40 large T-antigen. In K513-515A, lysines are mutated to alanines.

and Kutay, 1999) into and out of the nucleus. After reaching the nucleoplasm, import receptors bind the small GTPase Ran in the GTP-bound form (RanGTP) causing substrate dissociation. In contrast, nuclear export receptors bind substrate in the presence of RanGTP, forming a trimeric export receptor-substrate-RanGTP complex that crosses the nuclear pore and releases the substrate into the cytoplasm upon hydrolysis of RanGTP to RanGDP by the cytoplasmic Ran GTPase activating protein (RanGAP) (Melchior and Gerace, 1998; Weis, 1998; Gorlich and Kutay, 1999; Kuersten *et al.*, 2001).

The prevailing view is that protein localization is regulated by modulating the substrate: transport receptor interaction and the accessibility of substrate to the nuclear pore (Kaffman and O Shea, 1999; Turpin *et al.*, 1999). It is not known how glucocorticoids regulate GR intracellular localization. GR has two nuclear localization signals and one nuclear export signal (Picard and Yamamoto, 1987; Tang *et al.*, 1997; Black *et al.*, 2001). The first nuclear localization signal, NL1 (amino acids 497-524 of rat GR), is located within the DNA binding domain (DBD) and is similar in amino acid sequence to the SV40 NLS (Figure 1A). NL1 binds importin α2 in yeast two hybrid and GST-pulldown assays (Savory *et al.*, 1999). The second nuclear localization domain, NL2 (amino acids 540-795) resides in the ligand binding domain (LBD) of GR (Figure 1A). It fails to interact with importin α by GST-pulldown and yeast two hybrid analysis (Savory et al., 1999), and is otherwise poorly characterized.

In the simplest model, hormone binding might establish a GR conformation amenable to binding nuclear import receptors (Picard and Yamamoto, 1987; Pratt *et al.*, 1999). It appears, however, that importin α2 can bind to NL1 *in vitro* in the presence and absence of hormone (Savory *et al.*, 1999), implying that additional factors are required for the hormonal regulation of GR localization. As the NL2-containing region of GR conveys hormone-dependent localization to heterologous fusion proteins (Picard and Yamamoto, 1987), we hypothesized that hormone regulates GR localization by regulating

the interaction of NL2 with its nuclear import receptors. Thus, we screened in *S. cerevisiae* for nuclear import receptors that would bind NL2, tested whether mammalian homologues of those receptors could import GR *in vitro* and investigated the role of hormone in regulating the interaction with GR.

Chapter 1: Materials and Methods

Plasmid constructs:

pEGFP-N795 K513-515A was made from pEGFP-N795 (full-length rat-GR)(R. Sitcheran and Keith R. Yamamoto, unpublished) using Quick Change Mutagenesis (Stratagene). DNA fragments encoding the first N-terminal 525 amino acids of rat GR from pEGFP-N795 and pEGFP-N795 K513-515A were amplified with primers containing *XhoI* and *BglII* sites and subcloned into the *EcoRI*-Klenow-*BamHI* sites of pSG5 (Promega) making pSG5-N525 and pSG5-N525 K513-515. pEGFP-lacZ-540C is a fusion between β-galactosidase, GFP, and residues 540-795 of rat GR. The *lacZ* gene was amplified from pCMV-βgal (Spaete and Mocarski, 1985) and cloned into the *BglII* site of pEGFP-C1 (Clontech) producing pEGFP-*lacZ*. A DNA fragment encoding amino acids 540-795 of GR was amplified from pEGFP-N795 with primers containing *SalI* and *BglII* sites and cloned into the *SalI-BamHI* sites of pEGFP-*lacZ*.

pJW248, containing the *ADH1* promoter and GFP, was kindly provided by Jonathan Weissman (UCSF). pAdh-N795-GFP, wt or K513-515A, was constructed by excising full-length rat GR from pEGFP-N795 or pEGFP-N795 with *BamHI-XhoI* and ligating into the *SalI-BglII* sites of pJW248. pGPD-N795-GFP and pGPD-N795 K513-515A GFP (expressing GR from a GPD promoter) were created by excising GR-GFP from pAdh-N795-GFP or pAdh-N795-GFP-K513-515A with *BamH1-BglII* and subcloning into the *BamHI* site of pRS316-GPD-PGK, a kind gift from Anastasia Kralli (Scripps).

pYN525-GFP and pYN525 K513-515A GFP were created by amplifying a DNA fragment encoding amino acids 1-525 from pEGFP-N795 and pEGFP-N795 K513-515A using primers containing *XhoI* and *BamH1* restriction sites. The resulting fragment was subcloned into the *SalI-BglII* sites of pJW248. pY540C-*lacZ*-GFP is a fusion between

amino acids 540-795 of GR, β-galactosidase, and GFP. Fragments encoding residues 540-795 of rat-GR (540C) and the lacZ gene were amplified as described above and ligated into the *SalI-BglII* and *BglII* sites of pJW248, respectively. The pL2G3z GR lacZ reporter plasmid, (Bohen and Yamamoto, 1993), EB0347 expressing Pho4-GFP (Kaffman *et al.*, 1998b), pyxLhp1gfp expressing a fusion between Lhp1p-GFP (Rosenblum *et al.*, 1997), pPS1574 expressing Sxm1p, and pPS1117 expressing Sxm1p-GFP (Seedorf and Silver, 1997) have been described. pAdh-ER-GFP was constructed by amplifying the gene encoding ER α with primers containing *SalI* and *BamHI* sites and subcloning the resulting fragment into *SalI-BglII* sites of pJW248-GFP described above.

pGST-imp7 and pGST-imp8 were constructed by amplifying the inserts from pQE70 importin 7 and zz60 importin 8 (Gorlich *et al.*, 1997) and subcloning into pGEX4t-1 (Amersham). Plasmids encoding GST tagged mouse importin α2 GST-PTAC 58 (Imamoto *et al.*, 1995) and GST alone, pGEX 4t-1, have been described. For *in vitro* transcription, GR fragments were subcloned into pSG5 (Promega) that has an integrated T7 promoter. pSG5-N795 (rGR) (Darimont *et al.*, 1998) has been described. pSG5-540C was constructed by amplifying the DNA encoding amino acids 540-795 of pEGFP-N795 using primers containing *SalI* and *BglII*, and cloning the resulting fragment into the *EcoRI*/Klenow-*BglII* sites of pSG5.

For nuclear import substrates, a series of GFP-GST tagged import domains were constructed. To create pGST-GFP, the gene encoding GFP was amplified from pEGFP-C1 (Clontech) and subcloned into the *BglII* site of pGEX4t-1. Human SRP19a cDNA was amplified from HeLa cell total RNA using the Superscript One-Step RT PCR kit (Invitrogen) and subcloned into the *EcoRI-XhoI* sites of pGST-GFP generating pGST-GFP-SRP19a. A DNA fragment encoding residues 407-525 of rat GR was amplified from pEGFP-GR and subcloned into the *EcoRI-XhoI* sites of pGST-GFP creating pGST-GFP-407-525. Plasmid pGST-GFP-407-525 K513-515A was made from pGST-GFP-407-525

by Quick Change Mutagenesis. pGST-GFP-BIB was constructed by amplifying the DNA encoding the BIB domain from plasmid 4zL123a-cys (Jakel and Gorlich, 1998) using primers containing *EcoRI* and *XhoI* sites. Plasmid pGEX-2t-GFP-NLS was generously provided by Mary S. Moore (Baylor College of Medicine). For nuclear import assays, pQt Ran (pKW356), pQtRanQ69L (pKW592) (Nachury and Weis, 1999), and GST-PTAC97 (importin β) (Kose *et al.*, 1997) have been described. For nuclear export studies, pEGFP-N795 F463,464A was made from pEGFP-N795 (full-length rat-GR using Quick Change Mutagenesis (Stratagene). For the localization of different steroid receptor ligand binding domains, pEGFP^2 was constructed by amplifying the gene encoding GFP as above and subcloning into the *BglII* site of pEGFP-C1. pEGFP^2-GR LBD, pEGFP^2-AR LBD, pEGFP^2-ER LBD, pEGFP^2-MR LBD, and pEGFP^2-PR were constructed by amplifying the DNA sequence encoding the ligand binding domains of each steroid receptor with primers containing *SalI-BamHI* sites for ER and *SalI-BglII* for PR, AR, GR, and MR. The resulting fragments were ligated into the *SalI-BamHI* sites of pEGFP^2.

All constructs were verified by DNA sequencing. Klenow polymerase and all restriction enzymes were obtained from New England Biolabs.

Mammalian cell culture, transfections, and *in vivo* localization studies.

CV1, HeLa, and A549 cells were obtained from ATCC and cultured in Dulbecco's modified Eagle s Medium (DMEM) (Invitrogen) supplemented with 5% fetal bovine serum (FBS) (HyClone). For transient transfections, CV1 cells were split into 24 well plates (5 x 10^4 cells per well) on poly-DL-lysine (Sigma) precoated glass coverslips in phenol red-free DMEM (Invitrogen) supplemented with 5% charcoal stripped FBS (Picard and Yamamoto, 1987; Freeman and Yamamoto, 2001), approximately 16 hr before transfection. Cells were transfected with Lipfectamine 2000 (Invitrogen). Twenty four-48 hr after transfection, dexamethasone (Dex) (Sigma) was added to 1

μM for 1 hr. The cells were fixed in freshly prepared 4% paraformaldehyde (Sigma) in Phosphate Buffered Saline (PBS) for 20 min, and mounted onto glass slides in mounting medium (Citiflouor, Ted Pella Inc.) For hormone withdrawal studies, cells were washed extensively in PBS, and incubated in phenol red-free DMEM with charcoal stripped FBS for 24 hr before fixation.

For GFP-tagged proteins, localization was assessed by fluorescence microscopy with a Zeiss Axiovert S100 inverted microscope, 40x objective, and Spot digital camera. For immunofluorescence, fixed cells were permeabilized with PBS-0.1% Triton for 20 min and stained with the BuGR2 antibody (Gametchu and Harrison, 1984) in PBS/5% bovine serum albumen (BSA) to detect rat GR. Coverslips were washed three times with PBS, incubated with a goat anti-mouse rhodamine-linked secondary antibody (Molecular Probes) in PBS/5% BSA for 1 hr, and mounted on glass slides for microscopy. Within experiments, all images were taken using identical camera and microscope settings. All subcellular localization experiments were performed a minimum of 4 times.

Mammalian transcription luciferase assays were performed in 24 well dishes with U2OS cells. Briefly, cells were transfected with plasmids pSG5-N525 or pSG5-N525 K513-515A, pCMV-βgal (to control for transformation efficiency), and a GR luciferase reporter gene, pΔTAT3-Luc (Iniguez-Lluhi *et al.*, 1997). Luciferase activity was measured as previously described (Rogatsky *et al.*, 2001).

Yeast strains, handling, transformations, and localization studies:

For localization studies, W303-1a (Thomas and Rothstein, 1989) yeast were grown in YEPD or minimal SD media supplemented with appropriate amino acids and 2% glucose at 30°C unless otherwise noted. Yeast were transformed by the lithium acetate method (Gietz and Woods, 2002). For localization studies, overnight cultures in the appropriate SD medium in the presence or absence of 5 μM deacylcortivazol (Dac)

(Thompson *et al.*, 1981) were diluted to an OD595 of 0.1 and grown to an OD595 of 0.4 in the identical medium. Two µl of this suspension was spotted on a slide beneath a coverslip and protein localization was assessed by fluorescence microscopy with a 100x oil immersion lens on the Zeis microscope described above. For hormone withdrawal experiments, yeast were washed three times in SD medium lacking hormone and incubated at 30°C for 1 hr.

The localization of GR-GFP was monitored in fourteen yeast strains each deficient in a different nuclear transport receptor: CRMI (Stade *et al.*, 1997), CSEI (Xiao *et al.*, 1993), Kap95 (Iovine *et al.*, 1995), Kap104 (Aitchison *et al.*, 1996), Kap123 (Seedorf and Silver, 1997), LOS1 (Hopper *et al.*, 1980), MSN5 (Kaffman *et al.*, 1998a), MTR10 (Kadowaki *et al.*, 1994), NMD5 (He and Jacobson, 1995), PSEI (Seedorf and Silver, 1997), SRP1 (Yano *et al.*, 1992), SXM1 (Seedorf and Silver, 1997), Kap114 (Morehouse *et al.*, 1999; Pemberton *et al.*, 1999), and PDR6 (Titov and Blobel, 1999). Yeast strains PSY580, pse1-1, PSY1200, and PSY902 have been described (Seedorf and Silver, 1997). For temperature-sensitive strains, suspension cultures were grown overnight at permissive temperature, diluted to an OD595 of 0.1 and placed at the restrictive temperature. Pho4-gfp localization was performed as described (Kaffman *et al.*, 1998b).

For yeast hormone withdrawal experiments, yeast were handled as above for nuclear import. Yeast were checked by microscopy for nuclear localization. Yeast were then centrifuged and washed three times with buffer lacking hormone and then placed in new buffer containing or lacking hormone. After the indicated time, an aliquot of yeast was removed and localization was assessed by microscopy. For experiments with cycloheximide, after washing yeast were placed with 0.36 mM cycloheximide and hormone was removed.

Yeast reporter assays were performed as follows: Yeast were transformed with pGPD-N795-GFP (*URA)* or pGPD-N795-GFP K513-515A (URA), the pL2G3z (*LEU*)

reporter plasmid, and the pPS1117 SXM1-GFP (*TRP1*) rescue construct. Reporter assays with pYN525-GFP (*TRP1*) and pYN525 K513-515A GFP (*TRP1*) were performed as above with the pL2G3z (*LEU*) reporter plasmid. Colonies were grown to saturation at 25°C, diluted 1/40 into media with 5 μM Dac and grown to an OD595 between 0.2 and 0.3 for analysis on a Molecular Devices Therma Max plate reader.

Protein Expression and Purification:

GST, GST-imp7, GST-imp8, GST-PTAC 58, GST-PTAC97, GST-GFP, GST-GFP-BIB, GST-GFP-SRP19, GST-GFP-NLS, GST-GFP-407-525, and GST-GFP-407-525 K513-515A were expressed in BL21-Codon Plus (DE3)-RIL *E. coli* (Stratagene). Saturated overnight cultures in LB/Carb were diluted 1/100 to 1 liter and grown to an OD595 of 0.7 at 37°C. Cultures were then placed at 25°C for 20 min, induced with 0.4 mM IPTG for 7 hr, and harvested by centrifugation. Cell pellets were frozen, thawed, and resuspended in lysis buffer: PBS with 2 mM EDTA, 2 mM DTT and protease inhibitors mix (1 μg/ml leupeptin, pepstatin, aprotinin, and 1 mM PMSF) (Sigma). Lysates were incubated with 1mg/ml lysozyme (Sigma) for 30 min, sonicated, and clarified at 40,000Xg for 30 min, 4°C. For nuclear import substrates, GST-GFP, GST-GFP-Bib, GST-GFP-SRP19, GST-GFP-NLS, GST-GFP-407-525, and GST-GFP-407-525 K513-515A, cell pellets were resuspended in the above lysis buffer with 1% triton X-100. Proteins were purified according to (Moore and Schwoebel, 2000). For import assays, GST-PTAC 58 and GST-PTAC97 were purified according to (Kurisaki *et al.*, 2001) with the following variations: importin α2 or importin β were cleaved from GST using 10 units thrombin (Amersham) overnight at 4°C, eluate was passed over p-Aminobenzamidine resin (Sigma) to remove thrombin. The soluble protein was passed over a PD10 column (Amersham) equilibrated with 20 mM HEPES, pH 7.3, 110 potassium acetate, 0.5 mM EGTA, 2mM DTT, protease inhibitors, and concentrated on a Centricon 30 (Amicon).

Importin 7 was purified as in (Jakel and Gorlich, 1998) and importin 8 was purified as in (Dean *et al.*, 2001) with variations. Proteins were expressed in XL1-Blue cells (Stratagene), induced with 4 mM IPTG for 8 hr at 16°C at an OD595 of 0.8. After sonication, lysates were clarified at 40,000Xg for 30 min and purified over nickel agarose (Qiagen) in 50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM $MgCl_2$. Eluate was passed over a Superdex 200 gel filtration column (Pharmacia) and concentrated to 20 μM by a Centricon 30 concentrator. Ran-GDP and Ran-GTP Q69L were expressed in SG13 cells and purified according to (Nachury and Weis, 1999). Highly purified rat GR (N795) has been described (Perlmann and Wrange, 1988). Human importin β (Kutay *et al.*, 1997) was also induced in XL1-Blue cells as above. After clarification, importin β lysate was flash-frozen in liquid nitrogen.

Binding assays:

Lysates containing GST, GST-GRIP1, GST-importin 7, GST-importin 8, GST-PTAC 58 (importin α2), GST-GFP-SV40 NLS, GST-GFP-BIB, GST-GFP-407-525, and GST-GFP-407-525 K513-515A were thawed and incubated with 15 μL glutathione-agarose beads (Sigma) for > 1 hr at 4°C in binding buffer: 20 mM Tris, pH 8.1, 150 mM NaCl, 0.1% NP40, 14mM β-mercaptoethanol (β-ME) and protease inhibitors. GST beads were then washed 3 times in binding buffer containing 2 M NaCl.

For GST pull-downs with purified full-length GR, 15 μL glutathione beads bearing 4 μg bound GST fusion proteins were incubated with 150 ng purified rat GR overnight at 4°C in pull-down buffer: 20 mM Tris pH 8.0, 100 mM NaCl, 10% glycerol, protease inhibitors, 20 μg/ml BSA, 2 mM DTT, 1 mM EDTA, and 0.01% NP40. Beads were washed, bound proteins eluted as described previously (Darimont *et al.*, 1998), and immunoblotted with the BuGR2 antibody. For GST pull downs with *in vitro* transcribed-translated proteins, GR and truncation derivatives in the pSG5 vector were transcribed

and translated (TNT) using the Promega TNT kit as described (Darimont et al., 1998). Immobilized import receptors were incubated with 12 ng GR (as computed from the amount of incorporated [^{35}S] methionine) in binding buffer for 1 hr at 23°C. Bound proteins were washed 4 times with binding buffer containing 500 mM NaCl, eluted with SDS loading buffer, resolved by SDS-PAGE, and analyzed on a PhosphorImager (Molecular Dynamics Storm 820).

For GST pull-downs with immobilized nuclear localization domains, 4 μg immobilized import domain was incubated with 4 μg purified importin 7 or 72 μg HeLa extract overnight at 4°C in binding buffer. Bound proteins were eluted and resolved as described above and immunoblotted with a polyclonal importin 7 antibody (Gorlich *et al.*, 1997).

For binding experiments with recombinant RanQ69L, immobilized import receptors were pre-incubated with buffer or 40 μM RanQ69L for 1 hr at 4°C, before addition of 12 ng full-length GR for an additional 2 hr at 4°C, washed, and resolved as described (Darimont *et al.*, 1998).

For binding experiments with importin β, bacterial lysates containing 4 μg GST, GST-importin α, or GST-importin 7 were incubated with 15 μL glutathione-agarose beads overnight in pull-down buffer at 4°C in the presence or absence of lysate containing 4 μg human importin β. Beads were washed 3 times in pull down buffer and then incubated with 4 μg of GR fragment EX556 (Freedman *et al.*, 1989) containing NL1 for 3 hr at at 4°C. Bound proteins were resolved and immunoblotted with the BuGR2 antibody.

For hormone-dependent binding assays, immobilized import receptors or GRIP were mixed with 12 ng IVT full-length GR which had been translated in the presence or absence of hormone, as described (Darimont *et al.*, 1998). Bound proteins were resolved by SDS-PAGE and analyzed by PhosphorImager.

A549 extracts were made as described (Yamamoto *et al.*, 1974) with slight

modifications. A549 cells were grown in phenol red-free DMEM supplemented with 10% charcoal stripped FBS for three days. Cells from six confluent 15 cm plates were resuspended in an equal volume of lysis buffer 10 mM Tris, pH 8.1, 1 mM EDTA, 10% v/v glycerol, 50 mM NaCl, 1 mM β-ME, 100 μg/ml BSA, and protease inhibitors. Cells were lysed with 30 stokes of a Teflon homogenizer (Wheaton) and clarified at 20,000Xg for 10 min at 4°C. Typical extract concentration was 6-8 mg/ml. Immobilized importin 7, importin 8, importin α, or GST beads were incubated with 240 μg extract in the presence or absence of 100 nM dexamethasone for 30 min at room temperature. Bound proteins were washed and resolved as described (Darimont *et al.*, 1998). Gels were immunoblotted with GR-specific polyclonal antibody 283 (a kind gift of Michael Garabedian (NYU)).

Nuclear Import assay:

HeLa extract was prepared from 4 L of HeLa cells grown in suspension according to (Takizawa *et al.*, 1999) with modifications. The cell pellet was washed twice with PBS and once with 10mM HEPES, pH 7.3, 110 mM KOAc, 2 mM MgOAc, 2 mM DTT with protease inhibitors. Cell pellets were resuspended in 1.5 volumes 5 mM HEPES, pH 7.3, 10 mM KOAc, 2 mM MgOAc, 2 mM DTT, and protease inhibitors, and incubated on ice for 10 min. Cells were lyzed by Dounce homogenization with a type A pestle (20 strokes). After addition of 1/30 volume of 3M KOAc, homogenate was clarified at 23,500Xg for 20 min and centrifuged at 100,000Xg for 1 hr. Supernatant was dialyzed into transport buffer: 20 mM HEPES, pH 7.4, 110 mM KOAC, 2 mM MgOAC, 0.5 mM EGTA, protease inhibitors and 2 mM DTT and flash frozen in liquid nitrogen.

Nuclear import assays were performed as described (Adam *et al.*, 1990; Nachury and Weis, 1999; Takizawa *et al.*, 1999; Moore and Schwoebel, 2000). Cells were grown on poly-DL-lysine coated glass coverslips for 48 hr. Cells were washed twice with

transport buffer, incubated with 40 μg/ml digitonin in transport buffer for 6 min on ice, washed twice in transport buffer, and kept on ice for 10 min. Coverslips containing permeabilized cells were incubated for 30 min at 30°C or 4°C where noted on top of a 15 μL nuclear import reaction, which contained: 0.5 to 2 μM GFP tagged import substrate or 30 nM purified rat GR, 2 mg/ml HeLa extract, and an ATP regenerating system (1 mM ATP, 5 mM creatine phosphate, 10 units/mL creatine kinase). For ATP depletion, 2 units apyrase (Sigma) was added instead of the ATP regenerating system. After the import reaction, cells were washed twice in transport buffer, fixed in formaldehyde, and analyzed by immunofluorescence or direct fluorescence. For import assays with purified components, reactions contained 0.5 to 2 μM GFP-tagged substrate or 30 nM purified rat GR, 3 μM RanGDP, 2 μM RanBP7 or RanBP8 or 4 μM importin α2 + 1 μM importin β. For extract dilution experiments, HeLa extract was diluted to the designated concentration with transfer buffer and supplemented as noted with 2 μM RanBP7 or 4 μM importin α2. RanQ69L-GTP was added to the import reactions to 6μM as noted. All import experiments were performed a minimum of 4 times.

For experiments with immunodepleted nuclear extract, 140 μg HeLa extract was incubated with 2 μg importin 7 polyclonal antibody, control rabbit IGG (Santa Cruz Biotechnology) or buffer alone (mock) overnight at 4°C on a nutator. Mixture was then incubated with 25 μL protein A/G+ beads (Santa Cruz Biotechnology) for 90 min at 4°C on a nutator. Slurry was centrifuged at 500xG for one minute and the supernatent was then used for nuclear import assays. Twenty μL of the supernatent was resolved on a polyacrylamide gel and immunoblotted with the same polyclonal importin 7 antibody.

Chapter 1: Results

NL1 and NL2 are functional in yeast

GR is a transcriptional regulatory factor whose intracellular localization is controlled by its hormonal ligand (Figure 2A). We sought to identify nuclear import receptors that confer this redistribution. It has been previously shown that mammalian GR is functional when expressed in the budding yeast *Saccromyces cerevisiae* (Schena and Yamamoto, 1988). Similar to GR localization in mammalian cells, full-length GR was cytoplasmic in the absence of hormone, nuclear in the presence of hormone, and redistributed to the cytoplasm during hormone withdrawal in yeast (Figure 2a). Interestingly, the kinetics of GR nuclear import and export were altered between yeast and mammalian cells. Nuclear accumulation of GR ($t_{1/2}$ = 5 min) in mammalian cells was much faster than in yeast ($t_{1/2}$ = 7 hr). The kinetics of nuclear export was similarly altered. In yeast, GR export ($t_{1/2}$ = 30 min) was much faster than in mammalian cells ($t_{1/2}$ = 7 hr). While interesting, the cause of this kinetic difference was not investigated.

GR is a member of a 48 gene family of intracellular receptors that display a wide spectrum of localization phenotypes in mammalian cells. For example, nuclear accumulation of GR depends fully on ligand binding, whereas localization of the estrogen receptor (ER) is hormone-stimulated but not dependent, and the thyroid hormone and retinoid receptors are constitutively nuclear. To see if this was the case in yeast as well, we looked at the localization of ER-GFP in the absence of hormone. As observed in mammalian cells, ER-GFP was nuclear in the absence of hormone, while GR was cytoplasmic suggesting that the mechanism controlling this difference in localization is conserved between yeast and mammalian cells (Figure 2B).

In mammalian cells, nuclear localization depends on two domains, NL1 and NL2 (Figure 1). We found that truncated GR containing NL1 but not NL2 (N525)

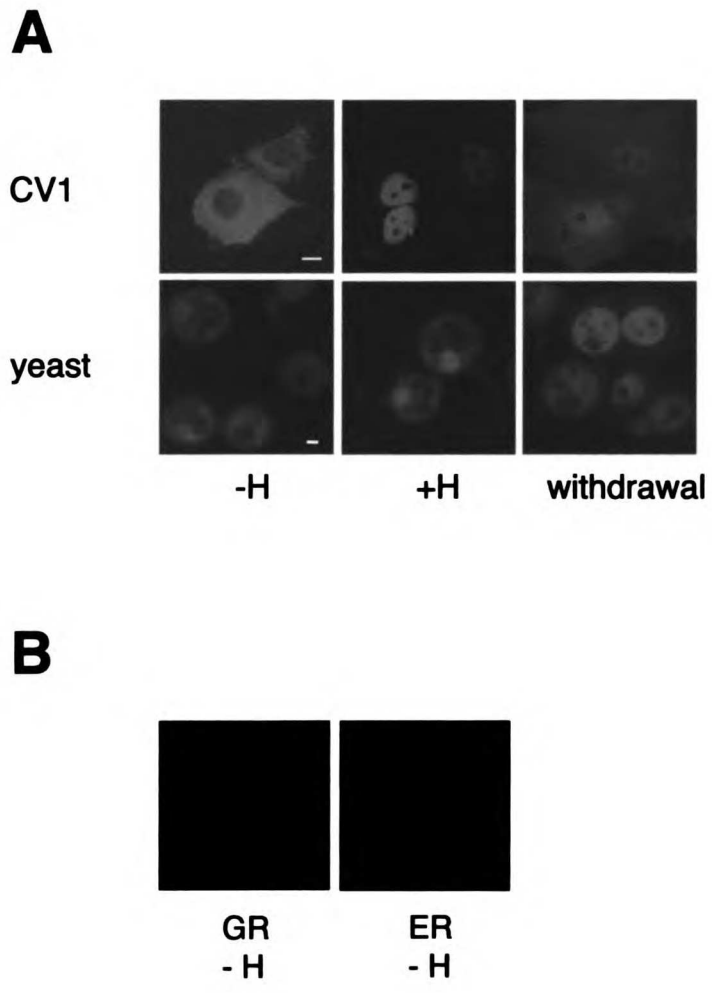


Figure 2: Hormone regulates GR but not ER localization in both yeast and mammalian cells. Bar, 10 μM for CV1 cell images, 1 μM for yeast images. (A) Nuclear localization of GR is conserved between yeast and mammalian cells. CV1 cells were transfected with pEGFP-N795. Twenty-four hours after transfection, cells were treated with EtOH (-H) or 1 μM Dex (+H) as indicated for 1 hr. For hormone withdrawal, hormone-treated cells were incubated overnight in media lacking hormone. W303-1a yeast were transformed with pAdh-GR-GFP and grown overnight in selective media containing EtOH (-H) or 5 μM DAC (+H) as indicated; yeast were diluted to an OD595 of 0.1 and grown to OD595 of 0.4 before microscopy. For hormone withdrawal, treated cells were incubated in media lacking hormone for 1 hr. The kinetics of GR nuclear transport are different in yeast than in mammalian cells. (B) Nuclear localization of ER is nuclear in the absence of hormone. Yeast were transformed with pAdh-GR-GFP or pAdh-ER-GFP as indicated. Localization was assessed by microscopy.

was nuclear in yeast even in the absence of hormone, consistent with the hormone independent localization of this fragment in mammalian cells (Figure 3). A fusion between NL2 (540C), GFP, and lacZ also displayed hormone-dependent localization in both mammalian and yeast cells (Figure 3). We replaced three of the conserved NL1 lysines with alanines (K513-515A) (Figure 1) by site directed mutagenesis. Mutations in these residues have been shown to abolish NL1 activity in mammalian cells (Savory *et al.*, 1999). As predicted, these mutations rendered the N525 K513-515A GFP fusion protein largely cytoplasmic (Figure 3). If the concentration of GR in the nucleus were significantly reduced, we would expect that the expression of a GR targets would be reduced as well. Indeed the mutations abolished the transcriptional activity of N525 in both mammalian and yeast transcription assays (Figure 4). However, corresponding K-> A mutations had only mild effects on the intracellular distribution of full-length GR (Figure 3). As previously observed in mammalian cells (Savory et al., 1999), the transcriptional activity of full-length GR K513-515A in the presence of hormone was modestly reduced (~2-fold) relative to wild type receptor (Figure 6). We conclude that GR import requires NL1 and NL2 in yeast.

Sxm1 is a nuclear import receptor for GR in yeast

We screened a panel of fourteen yeast strains with either null or temperature sensitive alleles of nuclear transport receptors, for those strains in which full-length GR K513-515A displayed altered localization (Materials and Methods). We found one strain, Δ *sxm1*, in which full-length GR K513-515A and NL2 (540C)-lacZ failed to accumulate in the nucleus in the presence of hormone (Figure 5). In addition, ectopic expression of Sxm1p was able to rescue this localization defect (Figure 5). A known substrate for Sxm1, Lhp1p (Rosenblum *et al.*, 1997), also failed to accumulate in the nucleus of Δ*sxm1 yeast* (Figure 5). N525 was nuclear in the Δ*sxm1* strain (Figure 5), demonstrating

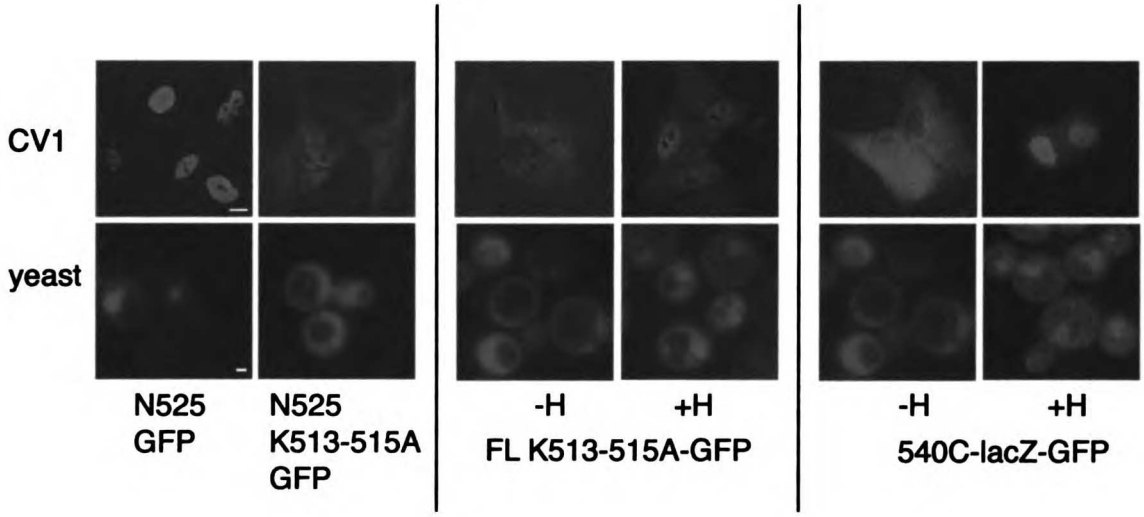


Figure 3: Import of GR is dependent on two nuclear localization signals (NL1 and NL2) in yeast and mammalian cells. Bar, 10 μM for CV1 cell images, 1 μM for yeast images. CV1 cells were transfected and yeast cells transformed with noted plasmids and treated with EtOH (-H), 1μM Dex (CV1 cells +H), or 5 μM DAC (yeast +H) as indicated. See materials and methods for constructs used.

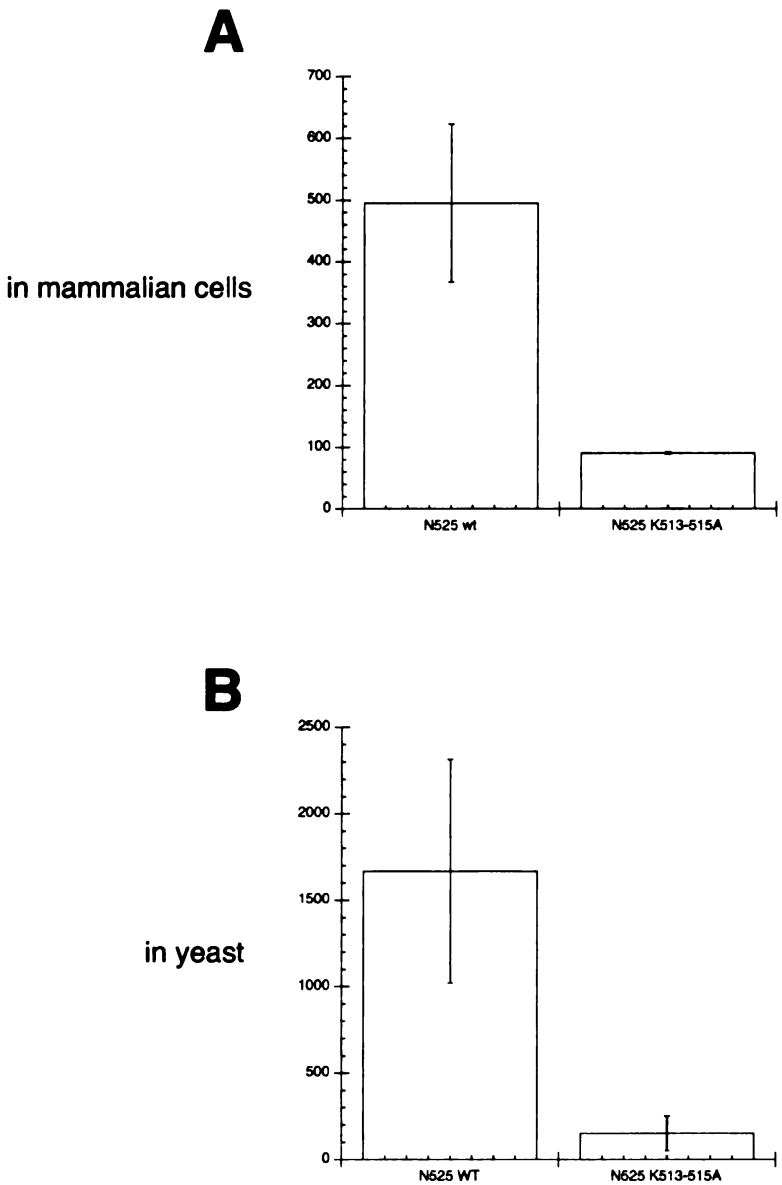


Figure 4: Residues K513-515 are required for N525 transcriptional activity in both mammalian and yeast cells. (A) Transcriptional activity of N525 and N525 K513-515A in mammalian cells. U2OS cells were transfected with plasmids pSG5-N525 or pSG5-N525 K513-515A, pCMV-βgal (to control for transformation efficiency), and a GR luciferase reporter gene, pΔTAT3-Luc. Luciferase activity was measured as previously described (Rogatsky *et al.*, 2001). (B) Transcriptional activity of N525 and N525 K513-515A in yeast. W303a yeast were transformed with plasmids pYN525-GFP or pYN525 K513-515A GFP and the pL2G3z (*LEU*) reporter plasmid, and lacZ expression was assayed with a plate reader (see Materials and Methods).

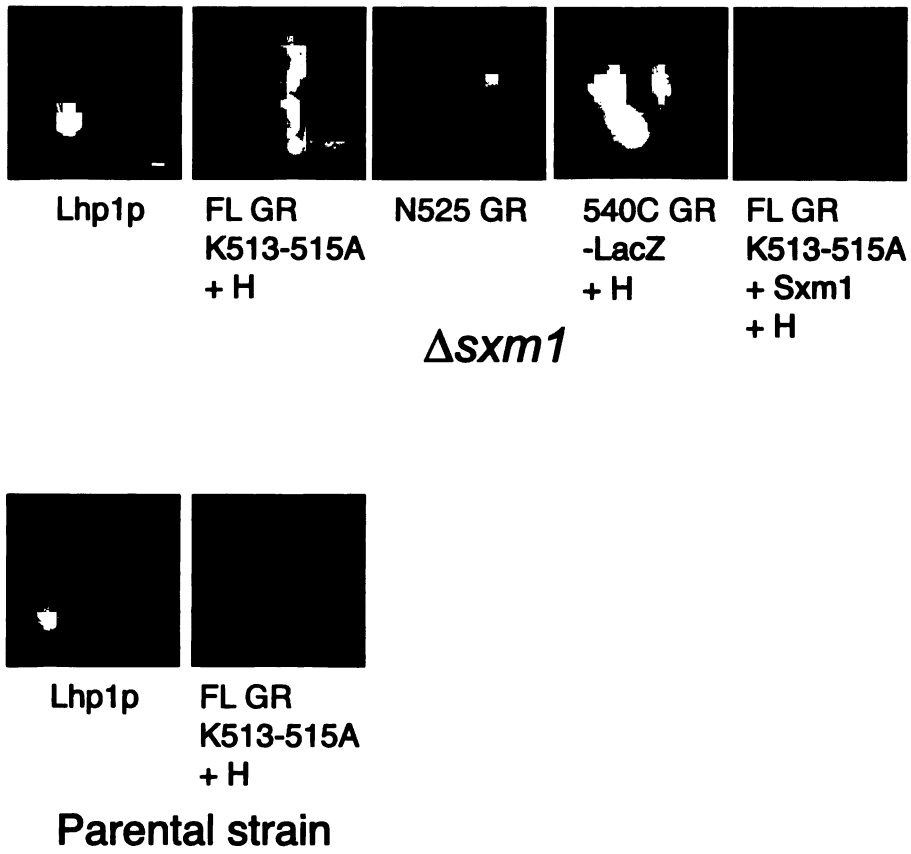


Figure 5: NL2 mediated import is altered in a Δ*sxm1* yeast strain. Bar, 1 μM. Plasmids encoding FL GR K513-515A-GFP, N525-GFP, 540C-lacZ-GFP, and Lhp1 were transformed into PSY1200 (Δ*sxm1* strain) or PSY902 (parental control); as noted. Plasmid pPS1574 was co-transformed to ectopically express Sxm1p and yeast treated with either EtOH (-H) or 5 μM DAC (+H).

that NL1-mediated import is not substantially affected in these cells. Finally, the transcriptional activity of a GR responsive reporter driving lacZ expression was reduced in the Δ*sxm1* strain, and this decrease in transcriptional activity could be rescued by ectopically expressed Sxm1p (Figure 6). Thus, Sxm1 is nuclear import receptor for GR in yeast.

N795 K513-515A localization was unaffected in an Δ*sxm1* strain constructed in the Blobel lab (Rosenblum et al., 1997) in a different strain background than PSY1200 (Seedorf and Silver, 1997) (Figure 7). These results suggest the presence of a genetic modifier, implying the existence of multiple import receptors for NL2 in some yeast strains.

A Yeast Model for GR Nuclear Export

We also asked if nuclear export was conserved between mammalian cells and yeast. To ensure that we were studying nuclear export as opposed to nuclear degradation of GR coupled with GR synthesis, we treated cells with cycloheximide before hormone withdrawal. Addition of cycloheximide did not alter the kinetics of nuclear export (Figure 8), suggesting that nuclear export of GR was in fact occurring. We screened the yeast transport receptor collection for strains in which GR export was altered. GR failed to export from the nucleus in Δ*MSN5* yeast, even six hours after hormone withdrawal (Figure 9). In contrast, GR had completely redistributed back to the cytoplasm in the parental yeast strain after 1 hr of hormone withdrawal (Figure 9). These data suggest that the MSN5 nuclear export receptor is critical for GR export in yeast.

We also looked at strains in which GR localization was altered in the absence of hormone. One strain was of particular interest in this regard. In Δ*los1* yeast, GR accumulated in the nucleus in the absence of hormone in yeast grown at 30°C and 37°C, yet localized in the cytoplasm of yeast grown at 25°C (Figure 10). Since the strain we

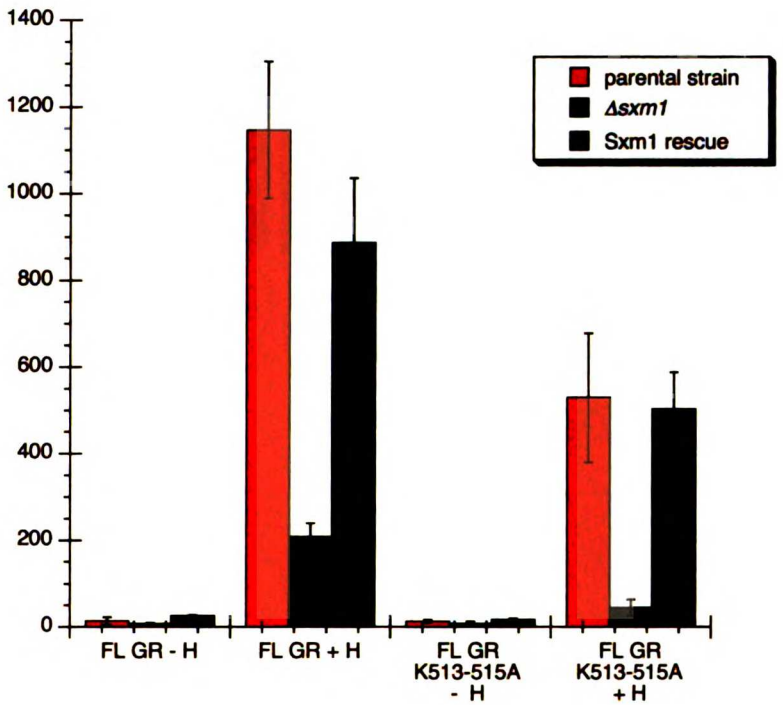


Figure 6: Transcriptional activity of GR is reduced in a *Δsxm1* yeast strain. PSY1200 (red bars) or PSY902 (gray bars) were transformed with pAdh-N795-GFP or pAdh-N795-K513-515A-GFP and the pL2g3z GR reporter plasmid. For ectopic Sxm1p expression (blue bars), PSY1200 yeast were transformed with above plasmids and pPPS1117 expressing Sxm1p. Data represent the average values of 6 independent assays.

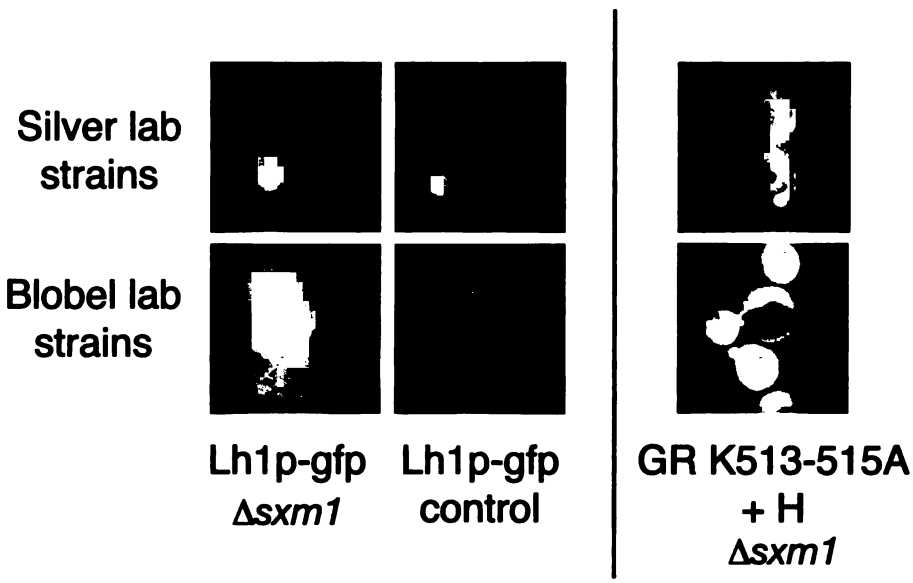


Figure 7: Strain specific differences between Δ*sxm1* yeast strains obtained from different laboratories. N795 K513-515A localization was unaffected in an Δ*sxm1* strain constructed in the Blobel lab (Rosenblum *et al.*, 1997) in a different strain background than PSY1200 (Seedorf and Silver, 1997). In contrast, the localization of Lh1p, a previously identified cargo for Sxm1 was altered in both strain backgrounds.

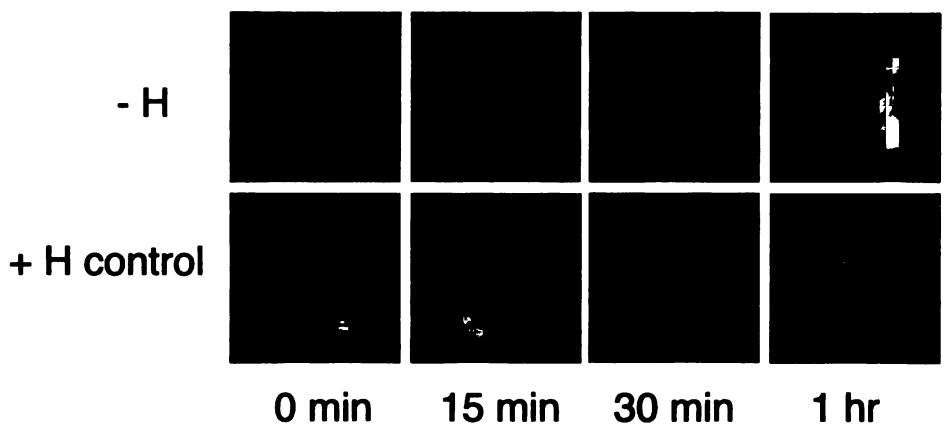


Figure 8: GR is rapidly exported during hormone withdrawal.
W303-1a yeast were transformed with pAdh-GR-GFP and grown overnight in selective media containing 5 μM DAC (+H); DAC (+H); yeast were diluted to an OD595 of 0.1 and grown to OD595 of 0.4 before microscopy. Cells were washed extensively in media lacking hormone, before being split into media containing cycloheximide in the presence or absence of hormone.

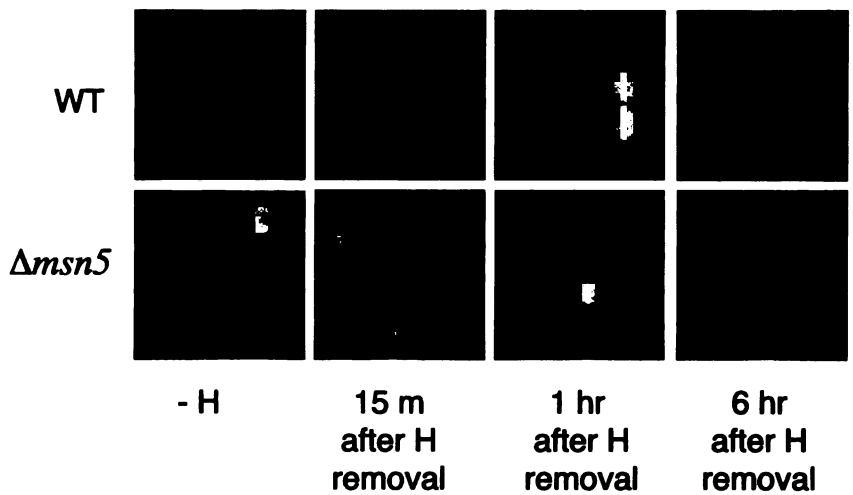


Figure 9: GR nuclear export fails to occur in *Δmsn5* yeast. Control and *Δmsn5* yeast strains (Kaffman *et al.*, 1998a) were transformed with pAdh-GR-GFP and grown in overnight selective media containing 5 μM DAC (+H) or EtOH as indicated; yeast were diluted to an OD595 of 0.1 and grown to OD595 of 0.4 before microscopy. Cells were washed extensively in media lacking hormone, and aliquots were taken at noted times for microscopic observation.

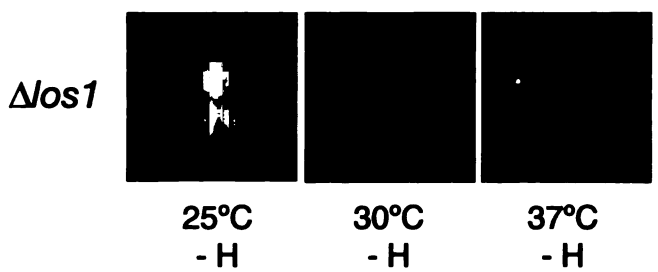


Figure 10: GR accumulates in the nucleus of Δ *los1* yeast in the absence of hormone at high temperature. Δ*los1* yeast (Feng and Hopper, 2002) were transformed with plasmid pAdh-GR-GFP. Yeast were grown at indicated temperature and localization was assessed by microscopy.

used lacked the Los1 protein, this temperature dependence suggests that Los1p is not a direct nuclear export receptor for GR. Los1p has been shown to be the nuclear export receptor for tRNA. Perhaps at higher temperatures the protein synthesis of a factor required to keep GR in the cytoplasm is affected, resulting in the altered GR localization observed. Alternatively, Los1 may be one of several nuclear export receptors required for GR nuclear export.

GR nuclear accumulation was not significantly altered in any of the other eleven strains (our unpublished results). For example, GR localization was unaffected in a *pse1-1* temperature sensitive strain, in contrast to the altered localization of Pho4, a known Pse1 substrate (Kaffman *et al.*, 1998b) (Figure 11).

Importin 7 and importin 8 bind directly to GR

The yeast Sxm1 protein has two mammalian homologues, importin 7 and importin 8, each of which have 23% identity and 43% similarity to Sxm1p (Gorlich *et al.*, 1997). We constructed fusions of both proteins to GST and tested whether they would directly bind to hormone-bound GR purified from rat liver (Figure 12). As expected, GST alone failed to bind GR under the conditions tested (lane 2), however GST-importin α, previously shown to interact with NL1, bound purified GR (lane 5). GST-importin 7 and GST-importin 8 also directly bound GR (lanes 3 and 4), suggesting that they may function as GR import receptors in mammalian cells.

Next, we mapped the regions of GR that interact with importin 7 and importin 8 by expressing ^{35}S-methionine labeled fragments of GR in reticulocyte lysate, and testing for their interaction with GST-tagged import receptors by coprecipitation (Figure 13). As expected, full-length GR bound importin α, importin 7, and importin 8 (lanes 3, 4, and 5). Importin α also interacted with N525 (containing NL1)(lane 8), but bound NL2 poorly (540C) (lane 13). These results are consistent with importin α serving as an import

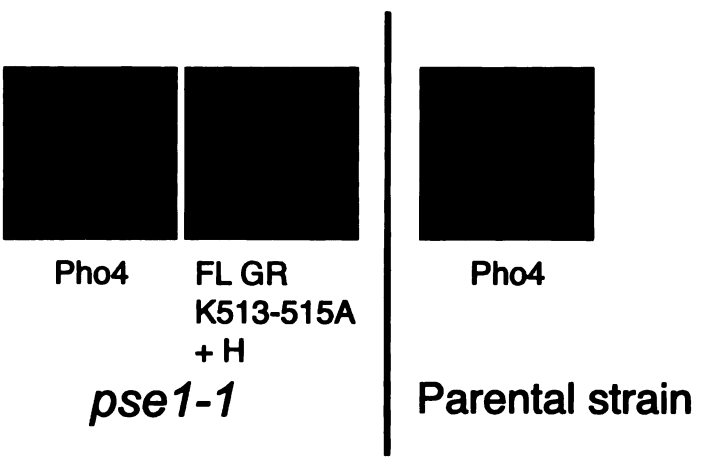


Figure 11: Localization of GR was unaffected in a *pse1-1* yeast strain. pAdh-N795 K513-515A-GFP and Pho4-GFP (EB0347) were transformed into *pse1-1*^{ts} or the control psy580 strain and localization was assessed as above.

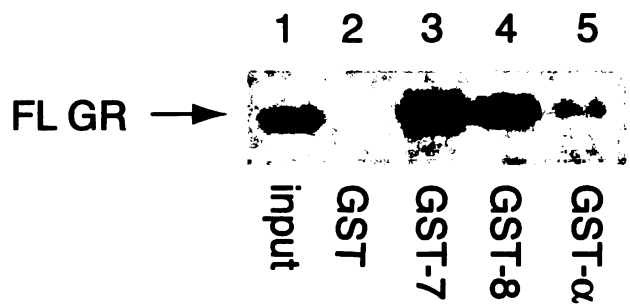


Figure 12: GR binds directly to importin 7, importin 8 and importin α. Input lane corresponds to 20% input. GST, GST-importin 7 (7), GST-importin 8 (8), or GST-importin α (α) (4 μg) were immobilized on glutathione resin and incubated with 150 ng of purified rat GR for 2 hr at 4°C. Bound proteins were resolved by SDS-PAGE and immunoblotted with rat GR-specific BuGR2 antibody.

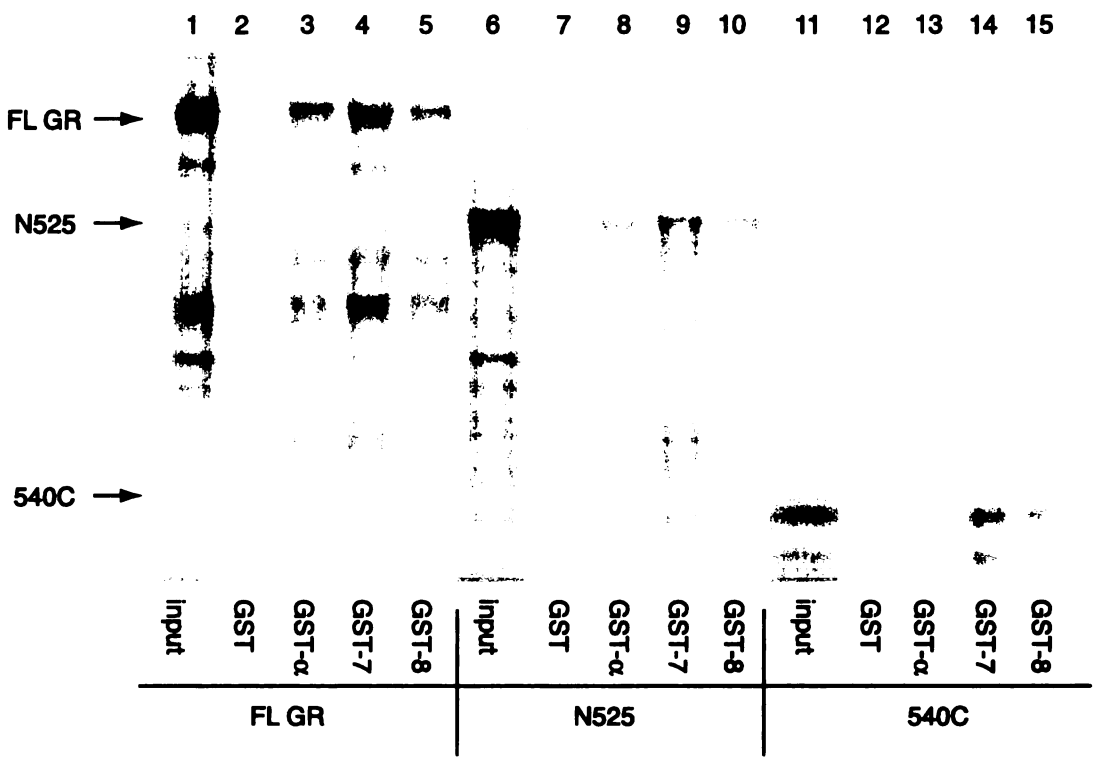


Figure 13: NL1 and NL2 of GR bind to importin 7 and importin 8. Input lane corresponds to 20% input. Immobilized GST, GST-importin 7 (7), GST-importin 8 (8), or GST-importin α (α) (4 μg) were incubated with 12 ng *in vitro* transcribed/translated N795, N525, or 540C for 1 hr at RT. Bound proteins were resolved by SDS-PAGE and detected by PhosphorImager.

receptor for NL1 but not NL2. Surprisingly, GST-importin 7 and GST-importin 8 bound both NL1 and NL2 (lanes 9, 10, 14, and 15), suggesting that there are at least two binding sites on GR for these import receptors.

We immobilized import substrates and tested their binding to purified importin 7. As a positive control, we used the beta-like importin binding (BIB) domain from the ribosome protein L123a that has previously been shown to bind importin 7 (Jakel and Gorlich, 1998). As expected, GST-BIB was able to bind purified importin 7 (Figure 14A, lane 4) and importin 7 only weakly bound to the SV40 NLS (Figure 14A, lane 3). In contrast, the GR 407-525 (containing NL1) bound purified importin 7 (Figure 14A, lane 5), and 407-525 K513-515A failed to interact (Figure 14A, lane 6) consistent with a requirement of NL1 for binding. Similarly, the BIB domain and the GR 407-525 fragment of GR bound to importin 7 in a HeLa cell extract (Figure 14B, lanes 4 and 5), and mutation of GR 407-525 to 407-525 K513-515A again abrogated this interaction (Figure 14B, lane 6). We conclude that GR can bind to both importin 7 and importin 8.

Finally, consistent with other importin 7 and importin 8 substrates (Jakel and Gorlich, 1998; Dean *et al.*, 2001; Jakel *et al.*, 2002), the presence of a GTPase deficient Ran mutant, RanQ69L, reduced the affinity of these import receptors for *in vitro* transcribed and translated full-length GR (Figure 15, lanes 5 and 7), suggesting that these importins may import GR. In addition, RanQ69L reduced the binding observed between GST-importin α and *in vitro* transcribed and translated full-length GR (Figure 15, lane 3). Importin α does not bind Ran directly (Rexach and Blobel, 1995), suggesting that another factor in reticulocyte lysate contributes to the observed binding between GR and importin α in the absence of RanQ69L. Since importin β binds importin α and the importin α/ importin β/SV40 NLS complex can be dissociated by RanQ69L (Rexach and Blobel, 1995; Gorlich *et al.*, 1996), we investigated the role of importin β in the binding of importin α to the NL1 of GR. The presence of recombinant importin β potentiated the

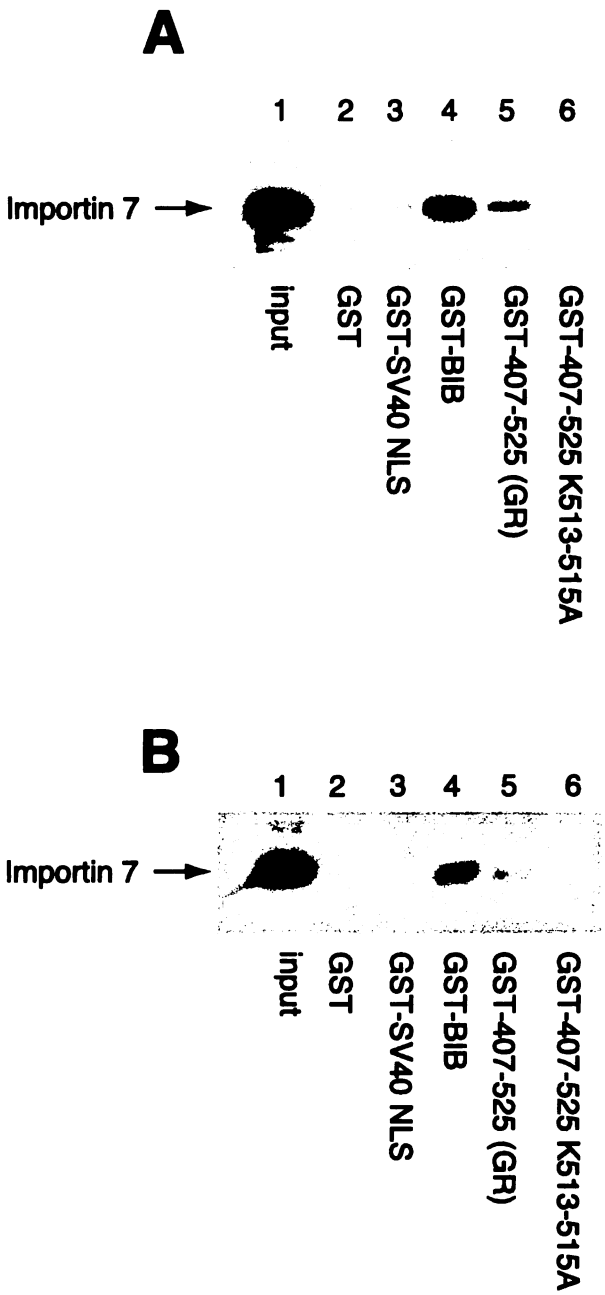


Figure 14: Immobilized GR NL1 binds to importin 7. (A) Immobilized GST-BIB, GST NLS, GST-407-525, GST-407-525 K513-515A, or GST alone (4 μg) were incubated with 4 μg of purified importin 7 overnight at 4°C. Bound proteins were resolved by SDS-PAGE and immunoblotted with a polyclonal antibody to importin 7. (B) Substrates described in (A) were incubated with 72 μg of HeLa extract overnight at 4°C and immunoblotted as above.

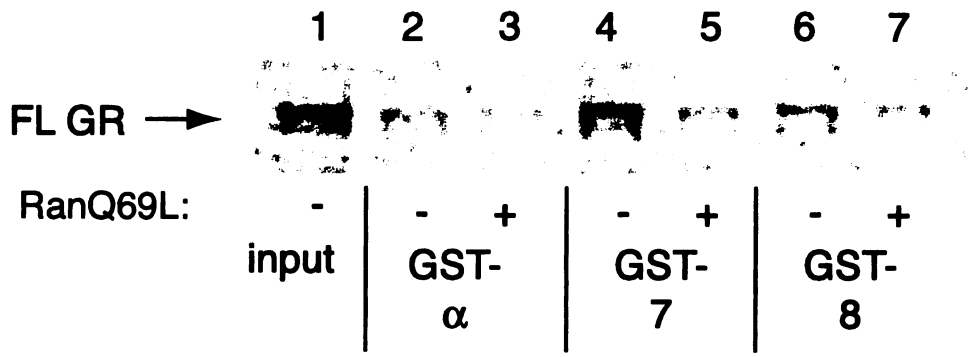


Figure 15: RanQ69L abrogates GR interaction with Importin α, Importin 7, and Importin 8. Immobilized import receptors (4 μg) were pre-incubated with 40 μM RanQ69L for 1 hr at 4°C and then incubated with 12 ng *in vitro* transcribed/translated full-length GR for 2 hr at 4°C.

binding of importin α but not importin 7 to a purified GR fragment (407-556) containing NL1 (Figure 16, compare lanes 3 to 4 and 5 to 6), suggesting that importin β plays an important role in importin α but not importin 7 mediated GR nuclear import.

Reconstitution of GR nuclear import *in vitro*

To determine if importin α, importin 7 and importin 8 can import GR, we used the digitonin permeabilized cell assay (Adam *et al.*, 1990). We first assessed nuclear import of a GR NL1-containing fusion protein, 407-525-GST-GFP, expressed and purified from bacteria. We found that nuclear import required HeLa cell extract and failed to occur on ice or in the presence of apyrase (Figure 17A), suggesting that energy is required for import. Next, full-length GR purified from rat liver was tested in the import assay. Since this protein is not tagged, we detected nuclear accumulation by indirect immunofluoresence. Import of full-length GR similarly required HeLa extract and ATP, and was not observed in the presence of apyrase or at 0-4°C (Figure 17B). Import of both GR 407-525 and full-length GR was abolished in the presence of RanQ69L, suggesting that Ran plays a role in the import of both the GR 407-525 fragment (Figure 17A) and full-length GR (Figure 17B).

Importin 7 and importin α/β can import GR *in vitro*.

Next, we attempted to reconstitute 407-525-GST-GFP import with purified components. As positive controls for our assay, we used the SV40 NLS, which is imported by importin α and β (Nigg, 1997), the BIB domain, which is imported by importin 7 (Jakel and Gorlich, 1998) and SRP19, which is imported by importin 8 (Dean *et al.*, 2001). Each was purified from bacteria as a protein fusion with GST and GFP. As observed previously by others, the import of each substrate required HeLa extract and ATP for import (Figure 18). In addition, as reported, a low level of import was observed

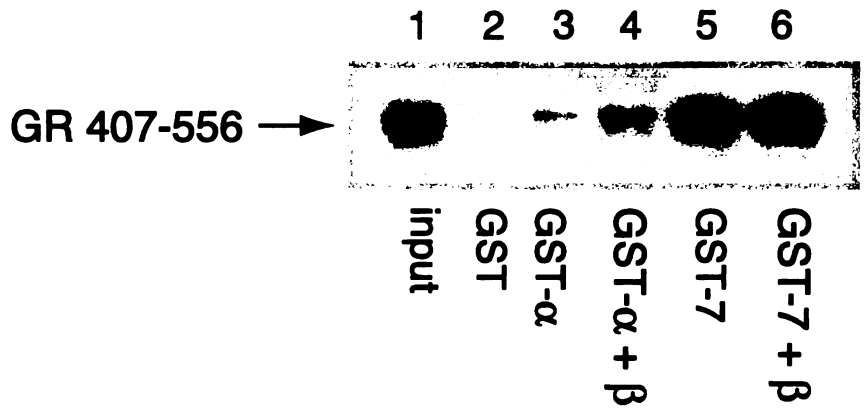


Figure 16: Importin β potentiates the importin α-GR interaction. 4 μg GST, GST- α, and GST-7 were immobilized on glutathione resin in the presence or absence of importin β overnight at 4°C. Bound import receptor complexes were incubated with 4 μg of a purified GR fragment (407-565) containing NL1 for 3 hr at 4°C.

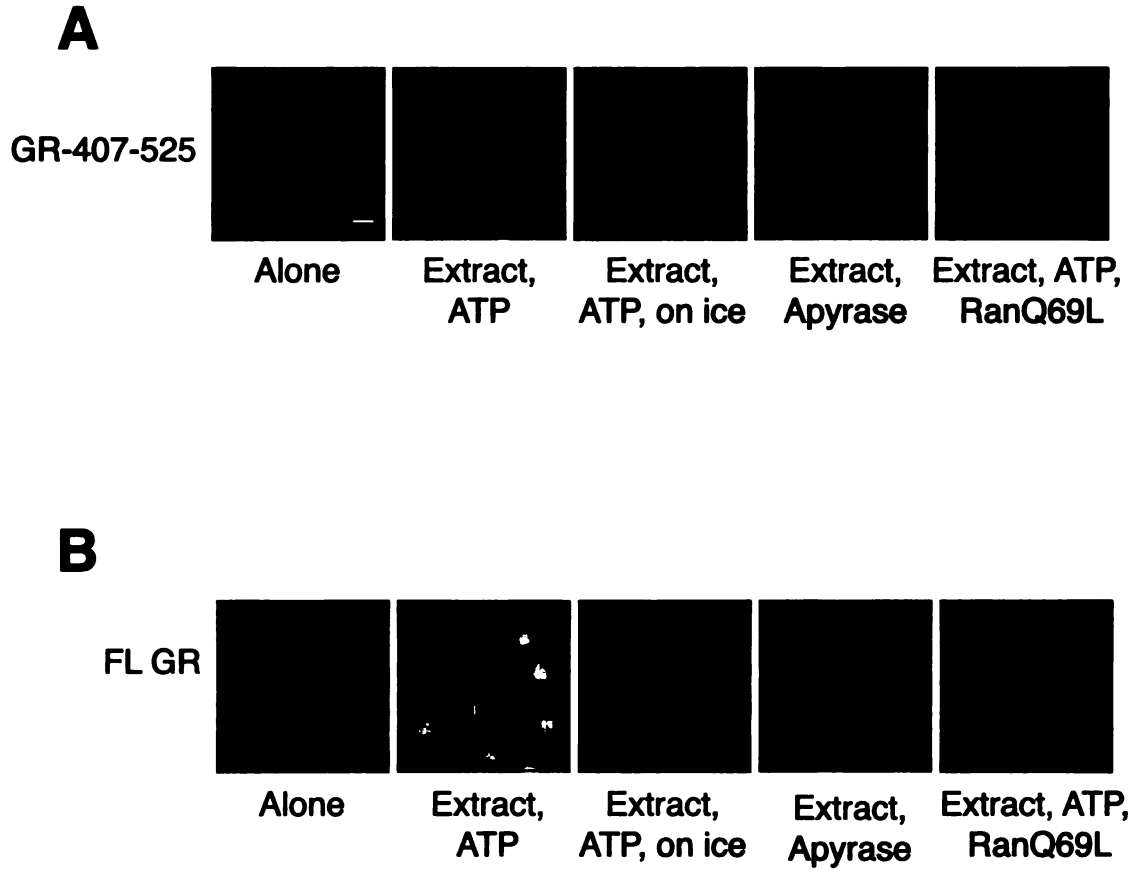


Figure 17: Reconstitution of GR nuclear import *in vitro*. Bar, 10 μM. (A) GFP-GST-407-525 (0.5 μM) was incubated with permeabilized cells, 2 mg/ml HeLa extract, ATP mix, 6 μM RanQ69L, or apyrase for 30 min at 30°C or 4°C, as indicated. (B) Purified GR (30 nM) was incubated with digitonin permeabilized cells, 2 mg/ml HeLa extract, ATP mix, 6 μM RanQ69L, or apyrase for 30 min at 30°C or 4°C, as indicated.

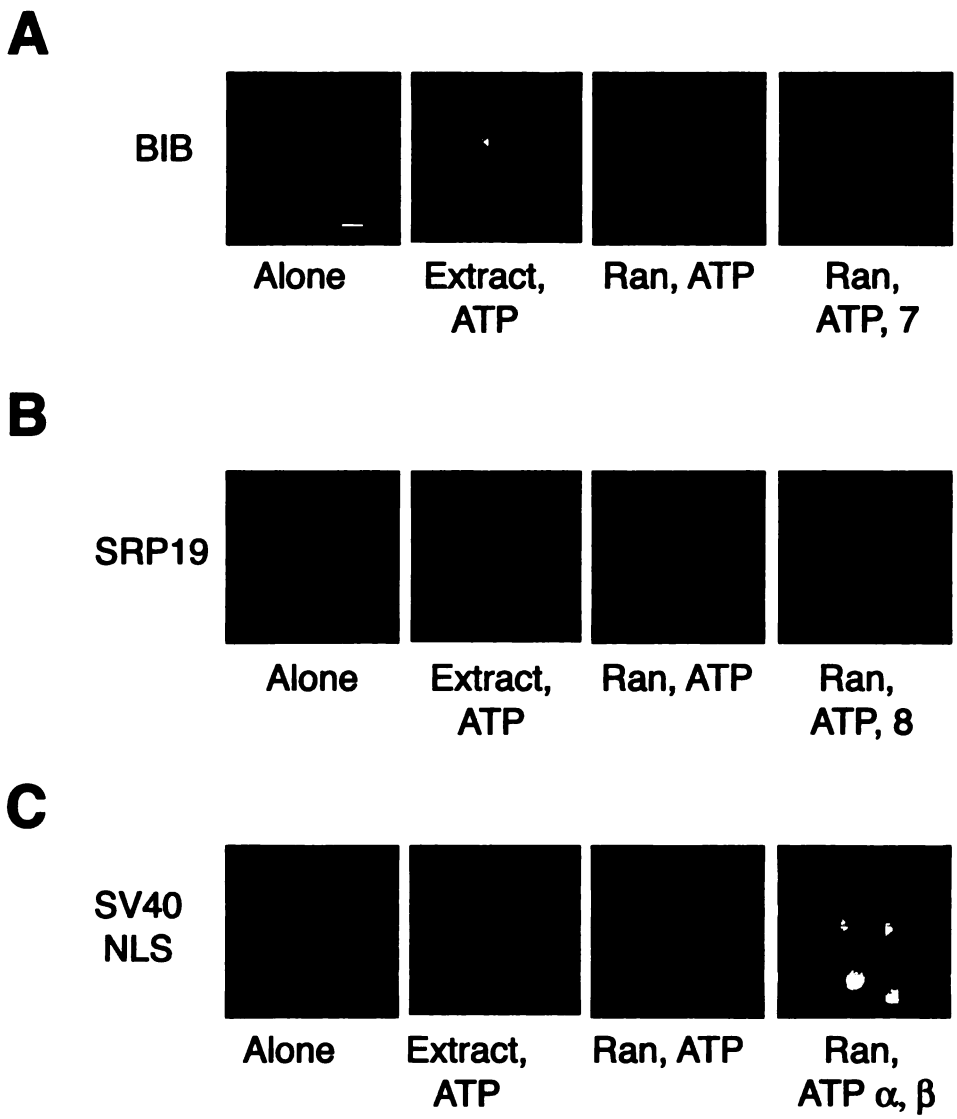


Figure 18: Positive controls for the digitonin nuclear import assay. Bar, 10 μM. BIB, SRP19, and SV40 import substrates (0.5 - 1 μM) and an ATP mix, 3 μM RanGDP, 6 μM RanQ69L, 2 μM importin 7, 2 μM importin 8, 4 μM importin α, or 1 μM importin β, or 2 mg/ml HeLa extract as indicated, were incubated with permeabilized cells for 30 min at 30°C.

in the presence of the small GTPase Ran-GDP and ATP (Jakel and Gorlich, 1998) (Figure 18), due presumably to the presence of residual import receptors in digitonin-permeabilzed cells. However, the import of the BIB domain was significantly stimulated by importin 7, SRP19 by importin 8, and SV40 NLS by the importin α -importin β heterodimer (Figure 18).

The import of the GR 407-525 was also stimulated by Ran-GDP and ATP (Figure 19). Addition of purified importin 7, or the importin α-importin β heterodimer significantly increased GR 407-525 nuclear import (Figure 19). Importin 8 failed to import GR 407-525 (Figure 19), although, it was able to import the SRP19 substrate (Figure 18B). Addition of RanQ69L abolished the nuclear import observed with purified import receptors (Figure 19), implicating Ran in the import of GR 407-525.

NL1 is strongly homologous to the SV40 NLS but shares little apparent similarity to either histone H1(o) or the BIB domain, previously identified as substrates for importin 7 (Jakel and Gorlich, 1998; Jakel *et al.*, 1999). We tested whether the residues required for import by importin α were also required for the import of GR 407-525 by importin 7. Mutation of three conserved NL1 lysine residues (407-525 K513-515A) abolished the ability of both the importin α–importin β heterodimer and importin 7 to import GR407-525 into the nucleus (Figure 20), suggesting that both classes of import receptors utilize NL1. Importin 7 failed to import the SV40 NLS in our assay, suggesting that although similar in sequence to the SV40 NLS, NL1 can be imported by additional import receptors (Figure 21).

We next performed the import assay with purified components and full-length GR. We observed no significant import of full-length GR with Ran-GDP, an energy regeneration system and either importin 7, importin 8, or the importin α-importin β heterodimer in any of the conditions we tried (Figure 22). To test if another factor in the extract was required for import of full-length GR, we supplemented import reactions with

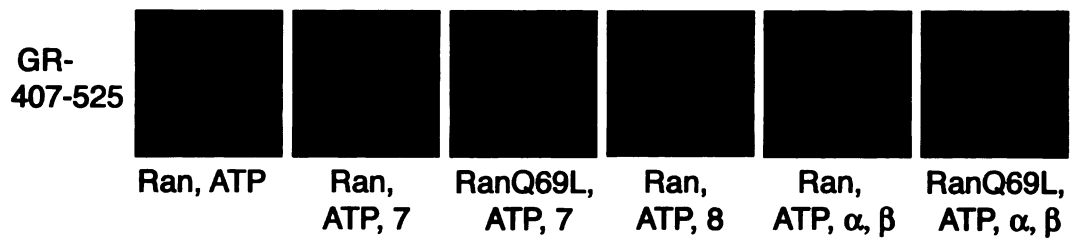


Figure 19: Importin 7 and the importin α-importin β heterodimer import GR 407-525 *in vitro*. Import assays were performed with 0.5 μM GFP-GST-407-525 and an ATP mix, 3 μM RanGDP, 6 μM RanQ69L, 2 μM importin 7, 2 μM importin 8, 4 μM importin α, or 1 μM importin β as indicated.

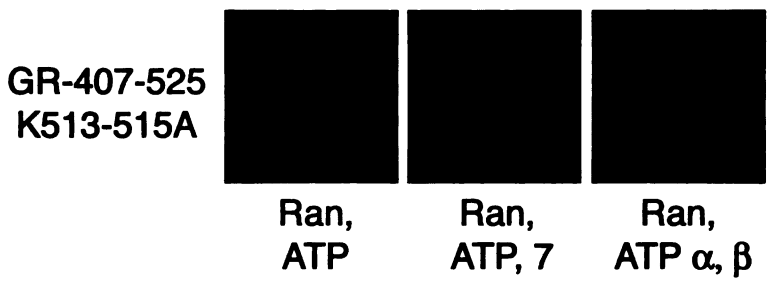


Figure 20: *In vitro* import of GR 407-525 requires NL1. Import assays were performed as above with 0.5 μM GFP-GST-407-525 K513-515A.

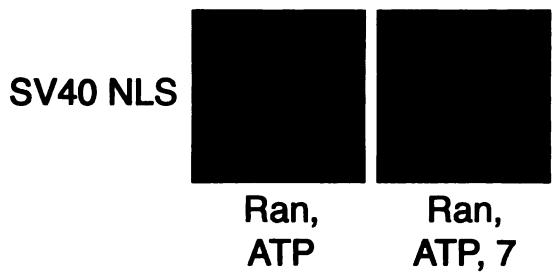


Figure 21: Importin 7 cannot import the SV40 NLS. Import assays were performed with 1 μM SV40 NLS and importin 7.

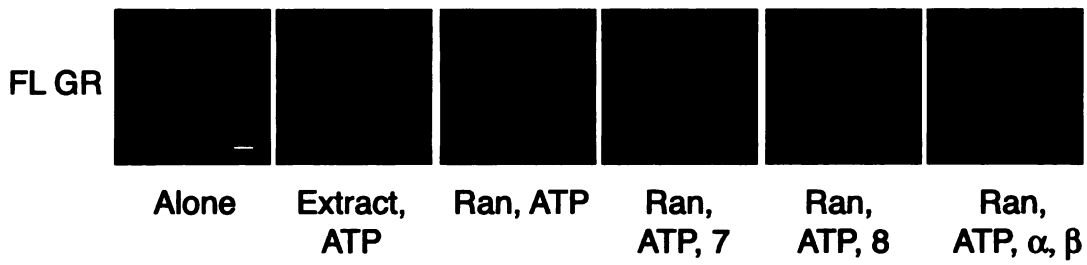


Figure 22: Importin 7 and the importin α-importin β heterodimer fail to import full-length GR *in vitro*. Bar, 10 μM. GR (30 nM), ATP mix, 3 μM RanGDP, and 2 μM importin 7, 2 μM importin 8, 4 μM importin α, or 1 μM importin β, as indicated, were incubated with digitonin permeabilized cells for 30 min at 30°C.

diluted HeLa extract. Using a concentration of HeLa extract that stimulated intermediate levels of import (Figure 23A), we found that addition of recombinant importin 7 and importin α, but not importin 8 potentiated the import of full-length GR (Figure 23B). This result suggests the presence of additional factor(s) in HeLa extract required for the import of full-length GR.

Importin 7 is not necessary for GR Nuclear Import in a HeLa lysate.

To investigate if importin 7 was necessary for the nuclear import of full-length GR in a HeLa lysate, we performed an immuno-depletion experiment using a polyclonal antibody specific for importin 7. As shown in Figure 24A, addition of the importin 7 antibody to HeLa lysate, but not a rabbit polyclonal antibody control, specifically reduced the concentration of Importin 7 found in a HeLa lysate. However, reducing the concentration of importin 7 found in the HeLa lysate had no effect on the amount of GR import observed from the lysate in the nuclear import assay (Figure 24B). This result suggests additional nuclear import receptors for full length GR in the HeLa lysate, consistent with our finding that the importin α - importin β heterodimer can also serve as an import receptor for GR (Figure 19).

Hormone is not required for GR to bind to import receptors

Finally, we tested the effect of hormone on the interaction of GR with importin 7, importin 8, and importin α. We could not readily test whether the interactions of purified full-length GR with nuclear import receptors were hormone dependent, as hormone is required for the purification of GR from rat liver (Perlmann and Wrange, 1988). Therefore, we examined these interactions using *in vitro* transcribed and translated GR in the presence and absence of hormone. As shown in Figure 25A, hormone did not affect the binding of importin α, importin 7, or importin 8 to full-length GR (lanes 5, 6, 7, 8, 9,

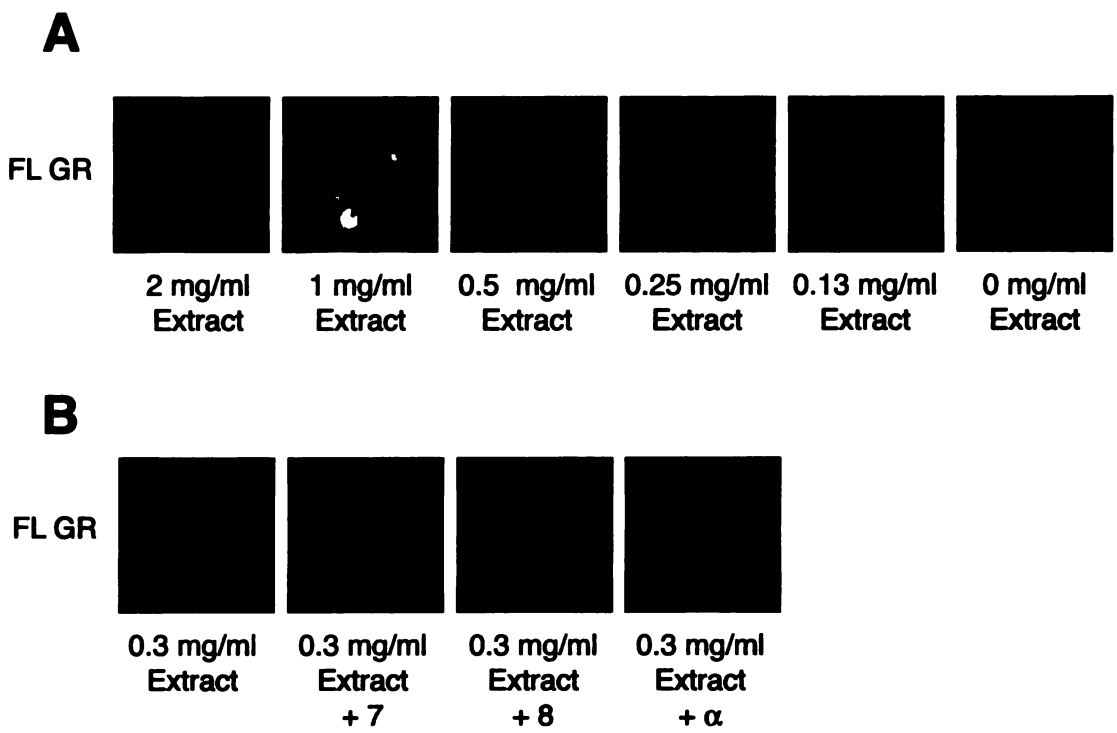


Figure 23: Importin 7 and the importin α-importin β heterodimer potentiate the import of full-length rat GR. Bar, 10 μM. (A) GR (30 nM) was incubated with permeabilized cells, ATP mix and the indicated concentration of HeLa cell extract. (B) GR (30 nM), ATP mix, 0.3 mg/ml HeLa extract and 2 μM importin 7 or 4 μM importin α were incubated with digitonin permeablized cells as above.

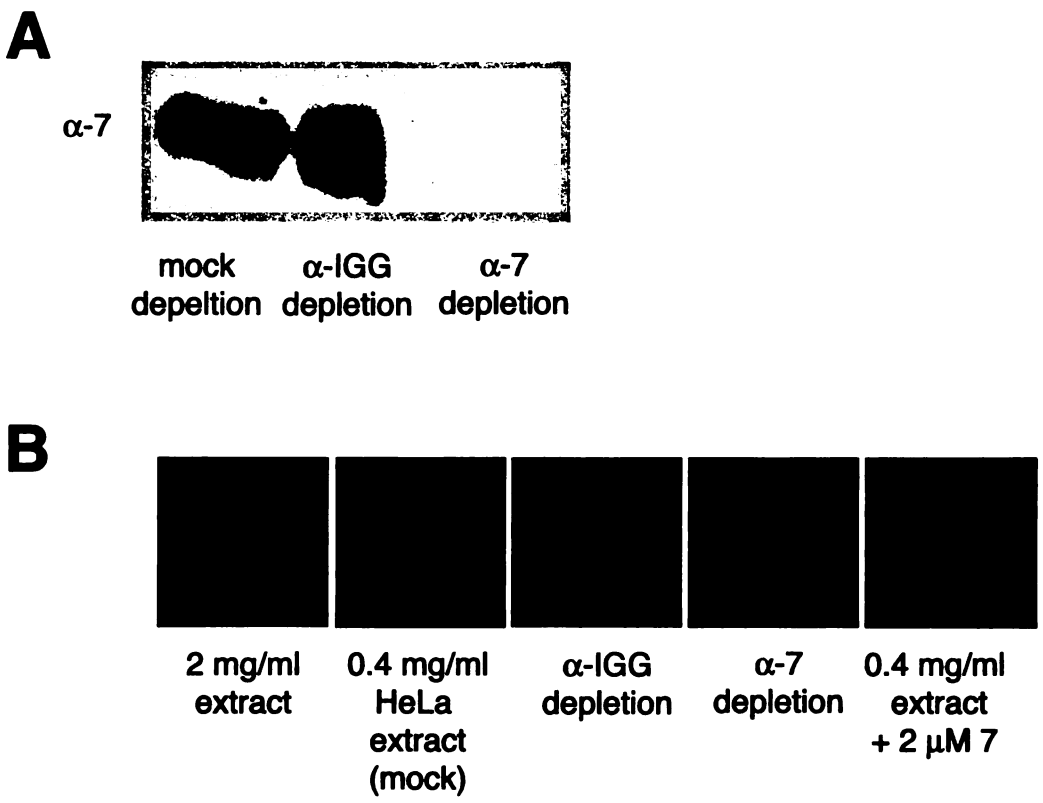


Figure 24: Importin 7 is not required for the import of full-length GR by a HeLa Extract. (A) Immunoblot showing the depletion of importin 7 from Hela Extract. Extract was immunoprecipitated with α- importin 7 (α-7) or α-igg antibody. Mock samples were incubated with buffer instead of antibody. (B) Nuclear import assay showing the nuclear import of 30 nM GR with 0.4 mg/mL lysates prepared in (A).

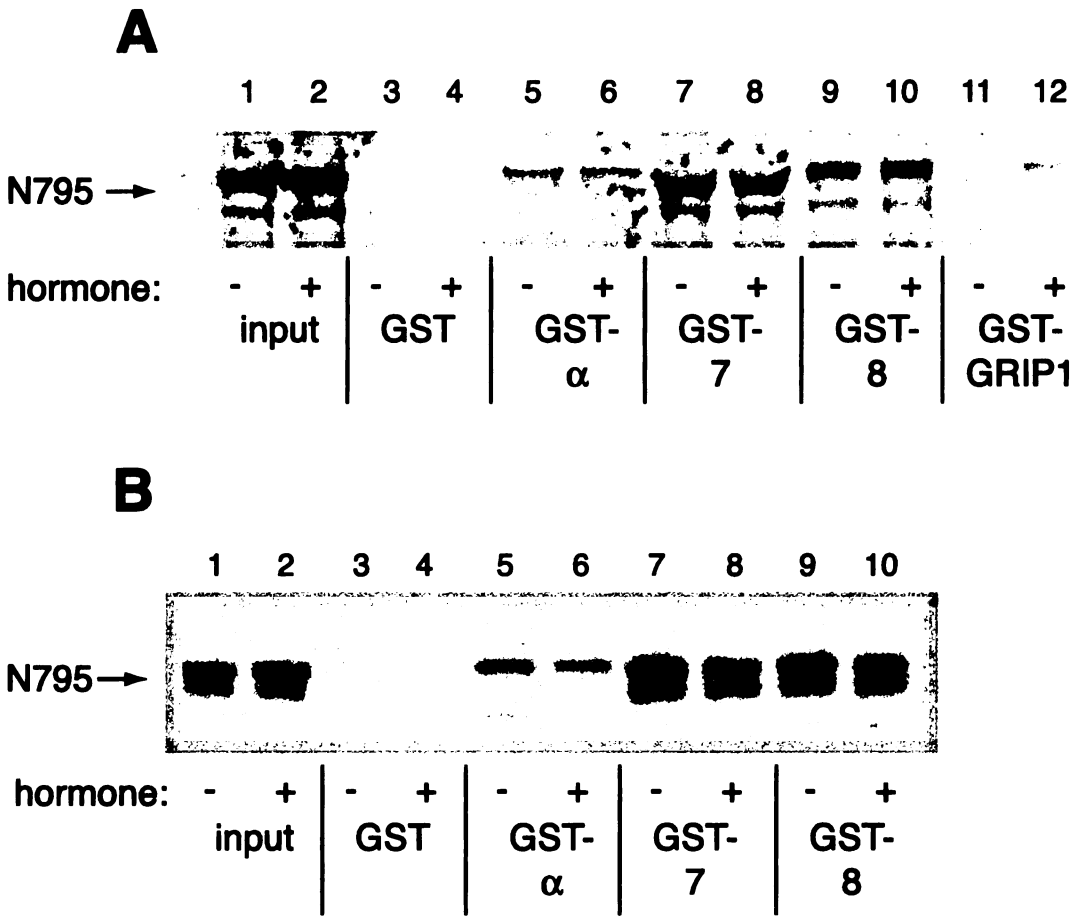


Figure 25: Interaction of importin 7, importin 8, and importin α with GR is hormone independent. Input lane corresponds to 20% input. (A) GST, GST-importin 7 (7), GST-importin 8 (8), GST-importin α (α) were immobilized on glutathione-agarose and incubated with 12 ng of ^{35}S labeled GR, which had been *in vitro* transcribed and translated in the presence or absence of 10 μM Dex. Bound proteins were resolved by SDS-PAGE and analyzed by PhosphorImager. (B) Immobilized import receptors were incubated with 240 μg of A549 cytosol in the presence or absence of 100 nM Dex for 30 min at room temperature. Bound proteins were resolved by SDS-PAGE and immunoblotted with the GR 283 polyclonal antibody.

and 10). As a positive control, we used GR interacting protein (GRIP1), a transcriptional cofactor previously determined to bind GR in a hormone-dependent manner (Darimont *et al.*, 1998). Indeed, hormone substantially stimulated the binding of GRIP1 to full-length GR (Figure 25A, lanes 11 and 12). Consistent with *in vitro* transcribed and translated GR, hormone did not affect the interaction of endogenous full-length GR from human A549 cells with GST tagged importin α, importin 7, and importin 8 (Figure 25B, lanes 5, 6, 7, 8, 9, and 10. These results suggest that hormone does not regulate the interaction of GR to its import receptor and thus must control GR intracellular localization in another way.

Chapter 1: Discussion

It has long been established that hormonal ligands direct the intracellular distribution of GR (Picard and Yamamoto, 1987). The GR LBD, which also houses NL2, conveys hormone dependent nuclear localization when fused to heterologous substrates (Picard and Yamamoto, 1987). However, technical problems with purifying functional recombinant GR have precluded biochemical identification of nuclear import receptors for NL2. We therefore took a genetic approach, screening a panel of *S. cerevisiae* strains to infer nuclear import receptors for GR NL2 in yeast, and then testing their vertebrate homologues. Indeed, this may be an efficient general strategy for identifying candidate import receptors for mammalian proteins.

Both NL1 and NL2 nuclear localization signals are functional in yeast, and localization of various GR truncations was similar in yeast and mammalian cells (Figure 1 and Figure 3). In yeast, a family of nuclear transport receptors homologous to importin β has been identified, and a set of strains constructed with individual disruptions in these genes (Kaffman *et al.*, 1998a; Kaffman *et al.*, 1998b). We tested NL2 mediated import in these strains and found that import was abolished in strains defective in Sxm1p (Figure 5).

Surprisingly, the mammalian homologues of Sxm1p, importin 7 and importin 8, bound both NL1 and NL2 of GR. Although NL2 has not been precisely mapped, the NL2 region lacks sequences homologous to NL1 or to any of the identified importin 7 and importin 8 substrates. NL1 is closely related to the well-characterized NLS sequences of SV40 T-antigen and nucleoplasmin (Figure 1; Picard and Yamamoto, 1987; Tang et al., 1997). NL1 binds importin α (Savory *et al.*, 1999); and Figure 12), which together with importin β confer nuclear localization in digitonin permeablized cells (Figure 19). In parallel experiments, NL1 also bound to importin 7 and importin 8 (Figure

12) and was imported by importin 7 (Figure 19). Whether importin α, importin 7, and importin 8 recognize distinct or identical residues within NL1 remains to be determined. Furthermore, we observed that importin β potentiates the binding of importin α to GR (Figure 16). Similar results have been observed for the SV40 NLS (Rexach and Blobel, 1995; Gorlich *et al.*, 1996). It will be interesting to determine the role that importin β plays in this interaction, whether it simply stabilizes the importin α-GR NL1 interaction or can bind to NL1 directly.

Gorlich and colleagues (Jakel *et al.*, 2002) have suggested that importin 7 and importin 8, among other import receptors, may bind exposed basic regions and act as cytoplasmic chaperones to prevent aggregation; conceivably, importin 7 and importin 8 might play a role in maintaining GR solubility during nuclear transport. It will be interesting to determine if importin 7 can import other proteins that carry NLSs similar to those on NL1 and the SV40 T-antigen (Figure 1), which are considered substrates of importin α. Notably, however, importin 7 failed to bind (Figure 14) or import (Figure 21) the SV40 NLS itself, demonstrating previously unrecognized selectivity within this class of nuclear localization motifs. Moreover, NL2 appears to be charge-neutral rather than basic yet bound importin 7 and importin 8 (Figure 13). How importin 7 and importin 8 recognize apparently distinct motifs, and whether they can simultaneously bind the same GR molecule remains to be determined. It is apparent, though, that multiple classes of nuclear import receptors can act on NL1.

Importin 7 alone and the importin α -importin β heterodimer were each competent to import an NL1-containing fragment of GR in an *in vitro* import assay in the presence of RanGDP and ATP (Figure 19), whereas they failed to import purified full-length GR (Figure 22). Addition of diluted HeLa cell extract to importin 7 or importin α permitted import of full-length GR (Figure 23), suggesting that components in the extract absent from the highly purified import system are required to reconstitute import. It is unlikely

that the diluted extract is simply providing additional importin 7 to the import reaction, for the amount of recombinant importin 7 in the reactions was $>10^3$-fold greater than that found in the diluted extract (our unpublished results). The observation that two separate import pathways could each import GR 407-525 but not full-length GR suggests that our biochemical import system lacks one or more general components required for GR import. Possible candidates include nuclear import machinery and molecular chaperones. Molecular chaperone complexes interact with GR and other intracellular receptors to facilitate hormone binding (Dittmar and Pratt, 1997) and the disassembly of GR-containing transcriptional regulatory complexes (Freeman *et al.*, 2000).

Importin 7 and importin 8 display 64% sequence similarity, and each is 23% identical to Sxm1p. We found that although both importin 7 and importin 8 could bind GR, only importin 7 could import GR. Such substrate specificity is reminiscent of SRP19 and RPL123a, which bind both import receptors but are imported only by importin 8 and importin 7, respectively (Jakel and Gorlich, 1998; Dean *et al.*, 2001). However, we cannot exclude the possibility that importin 8 might import GR under certain *in vitro* or *in vivo* conditions.

It is apparent from our work that multiple import receptors can transport GR to the nucleus (Figure 19 and Figure 23). Most other transcriptional regulatory factors examined seem to enter the nucleus via a single import pathway (Kaffman and O Shea, 1999), although certain non-transcription factor substrates for importin 7 and importin 8 can also be imported by other import receptors (Jakel and Gorlich, 1998; Jakel *et al.*, 1999; Dean *et al.*, 2001). Perhaps tissue-specific import-receptor expression modulates the relative rates or efficiencies of GR import in different target tissues, contributing to tissue specific responsiveness. It is also conceivable that different import receptors might be engaged when GR is activated by particular signals or combinations of signals.

During the course of our *S. cerevisiae* experiments, calreticulin was identified as an *in vitro* nuclear export receptor for GR (Holaska *et al.*, 2001). It is not clear how these results fit with our MSN5 data. Calreticulin does not have a clear yeast homologue. It is also not known if MSN5 binds to the GR Nuclear export sequence (Black *et al.*, 2001). The yeast nuclear export receptor MSN5 does have a mammalian homologue that has been shown to act as a nuclear export receptor for multiple substrates (Bohnsack *et al.*, 2002; Brownawell and Macara, 2002; Calado *et al.*, 2002; Gwizdek *et al.*, 2003; Yi *et al.*, 2003; Bohnsack *et al.*, 2004; Gwizdek *et al.*, 2004; Lund *et al.*, 2004). In the future, it will be interesting to determine if the mammalian homologue of MSN5 can bind and export GR from the nucleus.

In the absence of a bound agonist, the GR aporeceptor (apoGR) resides in a complex with molecular chaperones, competent to bind hormone, but inactive with respect to its other functions, such as DNA binding, regulatory cofactor recruitment, dimerization, and nuclear localization (Yamamoto *et al.*, 1974; Picard and Yamamoto, 1987; Dahlman-Wright *et al.*, 1990; Darimont *et al.*, 1998). Hence, in the simplest view, the maintenance of apoGR in the cytoplasm may reflect a failure of the aporeceptor to associate with import receptors. Indeed, localization of the yeast transcriptional regulator Pho4 is governed at least in part by phosphorylation dependent binding to the import receptor PseI (Kaffman *et al.*, 1998b); the binding of Smad3 to importin β is regulated by phosphorylation (Kurisaki *et al.*, 2001). However, we found that the interaction of GR with importin α, importin 7 or importin 8 is hormone-independent, suggesting that the aporeceptor complex includes import receptors. Thus, the hormone-regulated step for nuclear localization appears to be downstream of import receptor binding. For example, GR has been proposed to be rapidly exported from the nucleus in the absence of hormone, although evidence for this model remains controversial (Hache *et al.*, 1999; Savory *et al.*, 1999; Liu and DeFranco, 2000; Holaska *et al.*, 2001).

A nuclear export sequence has been identified in GR (Black *et al.*, 2001). However, in preliminary experiments, mutations in the NES shown to be important for nuclear export during hormone withdrawal had no effect on the localization of GR in the absence of hormone (Appendix 1). Additionally, a putative nuclear export sequence has been identified in a seventy-four amino acid region of the LBDs of AR, ER, and MR. This domain is sufficient to distribute GFP to the cytoplasm, and required for the cytoplasmic localization of AR in the absence of hormone (Saporita *et al.*, 2003). Whether the GR LBD also contains this domain remains to be determined. However, these provocative experiments suggest nuclear export may be playing an important role in the localization of steroid hormone receptors in the absence of hormone.

An alternative possibility is that GR may be tethered to a cytoplasmic factor or complex in the absence of hormone. Pratt and colleagues have demonstrated that components of the apoGR complex bind cytoskeletal factors (Galigniana *et al.*, 2001). Alternatively, (Kino *et al.*, 2003) have suggested that binding to the 14-3-3σ protein helps tether apoGR to the cytoplasmic compartment. Given these observations, it would be interesting to examine the intracellular distribution of GR in the absence of hormone by electron microscopy.

Our findings are also consistent with a third model, in which nuclear accumulation of GR may require the hormone dependent binding of a novel factor, perhaps corresponding to the putative component in the diluted HeLa cell extract required for the *in vitro* import of full-length GR. In the future, it should be possible to purify this factor by fractionating the extract and monitoring GR import activity.

At a minimum, our results suggest that the mechanism by which hormone regulates GR localization is more complicated than simply regulating the interaction of GR with other protein cofactors, as we hypothesized in the introduction of this chapter. In the future, it will be important to understand in detail how hormone transforms the

receptor, allowing it to enter the nucleus and regulate transcription. It seems likely that this transformation causes multiple changes in GR, dissociating GR from a complex of molecular chaperones, facilitating entry into the nucleus, binding to DNA and to transcriptional co-regulators. Our data demonstrate that the mechanisms of two elements of this transformation are different, as transcriptional co-regulators bind GR in a hormone dependent manner, whereas nuclear import receptors do not.

Future work could approach this question from two different directions. First, we have shown that GR is cytoplasmic in yeast in the absence of hormone, and localized to the nucleus in the presence of hormone (Figure 2). As nuclear accumulation is an early step in the transformation of GR by hormone, nuclear localization could be used as an effective screening tool. Indeed, the development of automated fluorescence microscopy systems (Huh *et al.*, 2003) should allow the identification of yeast factors required for the localization of GR in the cytoplasm in the absence of hormone and the identification of GR residues required for this cytoplasmic. Alternatively, the development of a mammalian RNA interference library in mammalian cells (Paddison *et al.*, 2004), in combination with automated fluorescence microscopy, could allow similar screens to be performed in mammalian cells. It will then be interesting to take the knowledge gained in these screens and use them to investigate other aspects of the hormone transformation of GR, such as DNA binding and transcriptional regulation.

Alternatively, investigators could take a reductionist approach and develop a simpler system to examine the hormone-mediated transformation of GR. The LBD of GR has been shown to regulate a wide variety of heterologues fusion proteins (Picard, 1993). Examining the mechanisms GR uses to directly regulate some of these heterologous fusion proteins, such as kinases or recombinases with defined structures and *in vitro* activity assays should give insights into the nature of hormonal control that can then be applied to LBD function in NRs.

GR is a member of a 48-gene family of NRs that displays a wide spectrum of localization phenotypes. For example, nuclear accumulation of GR, AR, and MR depends fully on ligand binding, whereas localization of the estrogen receptor is hormone-stimulated but not dependent, and the thyroid hormone and retinoid receptors are constitutively nuclear. It will be interesting to determine whether other members of the intracellular receptor family serve as importin 7 substrates, if they require the putative factor employed by GR for nuclear import, and the hormonal requirements for their interaction and import. In preliminary data, NL2 function is conserved in AR, MR, GR, and PR but not in ER (Appendix 1). A detailed understanding of the molecular features governing the nuclear localization of these receptors would provide insight into the physiological rationale for the observed differences in localization. Furthermore, intracellular receptors are clinically important, the targets of many prescribed pharmaceuticals. For example, AR plays a critical role in prostrate cancer pathogenesis. Androgen receptor antagonists are widely prescribed to patients diagnosed with prostrate cancer. However, over time, these same antagonists become AR agonists through a poorly understood mechanism, and actually make the cancer worse (Heinlein and Chang, 2004). Identification of the mechanisms regulating AR intracellular localization may allow the development of novel compounds regulating AR localization, preventing AR entry into the nucleus and thus helping patients with prostrate cancer.

Chapter 2: Development of a pharmacological tool to study *C. elegans* sterol metabolism.

Chapter 2: Abstract

Sterols are required for normal growth and reproduction in *C. elegans*. In order to investigate the mechanisms by which sterols regulate worm physiology, we tested the effects of small molecule inhibitors of mammalian steroid biosynthesis on worm growth, reproduction, and lifespan. Among those studied, a class of imidazole based p450 inhibitors: ketoconazole, miconazole, sulconazole, and clotrimazole significantly inhibited *C. elegans* growth and reproduction, and caused defects in gonadal migration (mig). These phenotypes are similar to those found in worms grown on cholesterol-deprived media, and the drug phenotypes are more severe in the absence of cholesterol. The gonadal migration defect suggested ketoconazole might affect a putative endocrine pathway containing a nuclear receptor, *daf-12*, which regulates both gonadal migration and dauer formation. Indeed, null alleles of *daf-12* suppressed the ketoconazole mig phenotype but not the growth and reproductive phenotypes, suggesting that ketoconazole affects cholesterol metabolites important for multiple signaling pathways.

Chapter 2: Introduction

Under optimal conditions, *C. elegans* develops from eggs through four larval stages (L1-L4) to egg-laying adults in four days (Figure 1). Cholesterol is required for several aspects of normal worm physiology, however it cannot be synthesized by the organism (Hieb and Rothstein, 1968). Rather, it must be harvested from the environment. Worms grown on plates lacking cholesterol within a single generation show delayed development and reduced progeny, and inappropriate dauer (see below) formation (Yochem *et al.*, 1999; Gerisch *et al.*, 2001; Jia *et al.*, 2002; Shim *et al.*, 2002; Merris *et al.*, 2003). By the next generation in the absence of cholesterol, worms display molting defects, larval arrest, and further decreased fertility.

One important question is how cholesterol regulates worm biology. In mammalian cells, cholesterol plays a major structural role in cell membranes and is also important for many signaling pathways. In *C. elegans,* cholesterol is likely required for signaling, rather than cell structure. First, cholesterol is not found uniformly in all *C. elegans* tissues; investigations with the naturally fluorescent cholesterol analogue DHT and fluorescent cholesterol binding compound filipin show that cholesterol has a tissue specific distribution. It is found in the pharynx and intestine, nerve ring, excretory gland cell, and germ cells, but not in other *C. elegans* tissues and organs (Matyash *et al.*, 2001; Merris *et al.*, 2003). Second, worms display development and reproductive defects when grown on a cholesterol enantiomer (Crowder *et al.*, 2001). Such an enantiomer would presumably maintain its membrane structural role, but would likely be unrecognized by cholesterol metabolizing enzymes, due to its difference in chirality. These experiments suggest that the *C. elegans* specifically requires cholesterol metabolites for normal development and reproduction. Finally, the gene encoding *let-767* was recently cloned and it encodes the *C. elegans* homologue of 17β-HSD3, an enzyme crucial for the

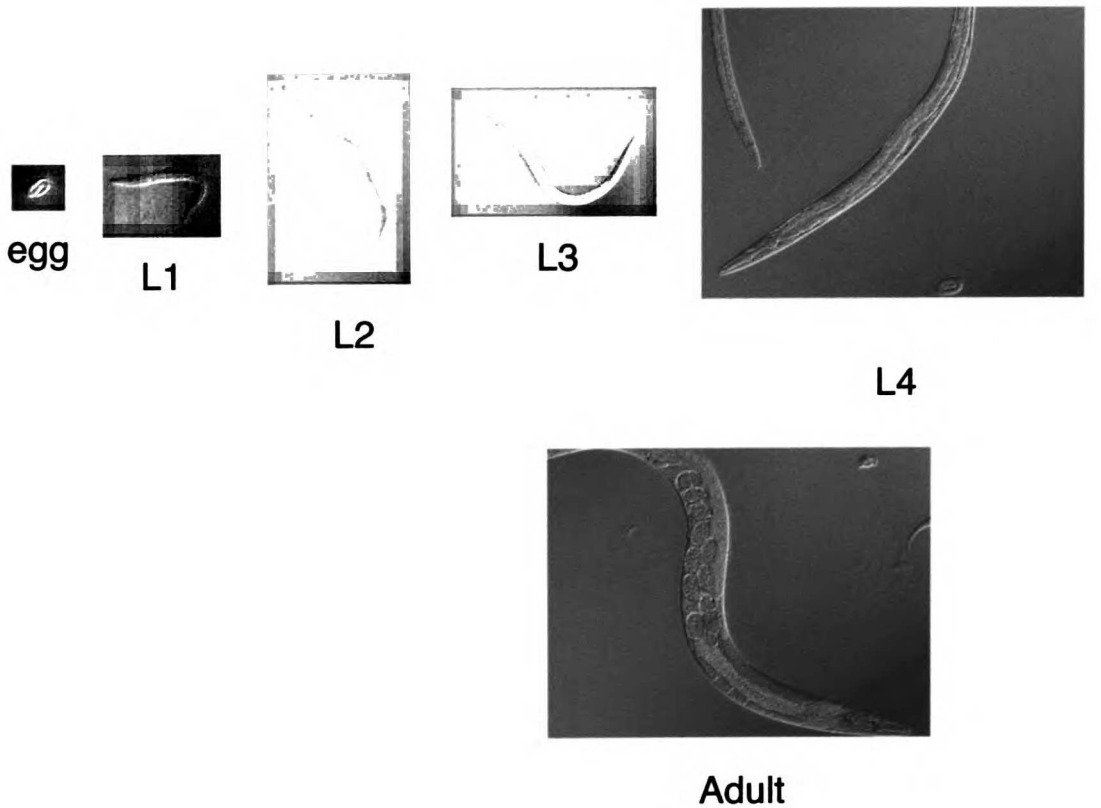


Figure 1: Worms develop from eggs through four larval stages (L1-L4) to adults. Worms were grown on NGM-lite plates supplemented with cholesterol, seeded with OP50 bacteria and photographed on a Zeiss Microscope with a 25x objective.

conversion of androstenedione to testosterone in the testes of mammals (Kuervers *et al.*, 2003). Let-767 worms displayed growth, reproduction, and molting defects, similar to phenotypes observed in worms grown on plates lacking cholesterol. Furthermore, cholesterol depletion potentiates these phenotypes. Thus, it seems likely that cholesterol metabolites play important roles in *C. elegans* biology.

Cholesterol plays a role in two main types of signaling in other organisms. Cholesterol modification of Hedgehog proteins is required for their normal function in *Drosophila* (Jeong and McMahon, 2002). Several families of proteins with Hedgehog-like cholesterol binding domain have been identified in *C. elegans* (Aspock *et al.*, 1999), along with a homologue of the patched hedgehog receptor (Kuwabara *et al.*, 2000). Cholesterol also serves as a precursor to a wide variety of small molecule metabolites and signaling molecules in mammalian cells, including nuclear receptor ligands. *C. elegans* has over 280 nuclear receptors (Van Gilst *et al.*, 2002; Gissendanner *et al.*, 2004), most with predicted structural folds similar to that of mammalian steroid hormone receptors (Brelivet *et al.*, 2004). Additionally, *C. elegans* has approximately 80 cytochrome p450 enzymes (Gotoh, 1998; Nelson, 1998); homologues of these p450 enzymes synthesize or metabolize sterols in mammals.

Furthermore, recent experiments have suggested that a cholesterol metabolite is involved in the regulation of a crucial *C. elegans* developmental decision (Figure 2A). Under conditions of stress—high temperature or lack of food, L2 worms can enter an alternative dauer life stage instead of progressing to L3 (Cassada and Russell, 1975; Golden and Riddle, 1984) (Figure 2a). Worms can stay in the dauer life stage for months until conditions improve, at which point they develop into normal adults (Riddle, 1997). Epistasis analysis places a nuclear receptor, *daf-12* distal in this signaling pathway. Both dauer defective (Daf-d) and dauer constitutive (Daf-c) *daf-12* alleles have been isolated, suggesting that *Daf-12* plays a critical role in regulating this life decision.

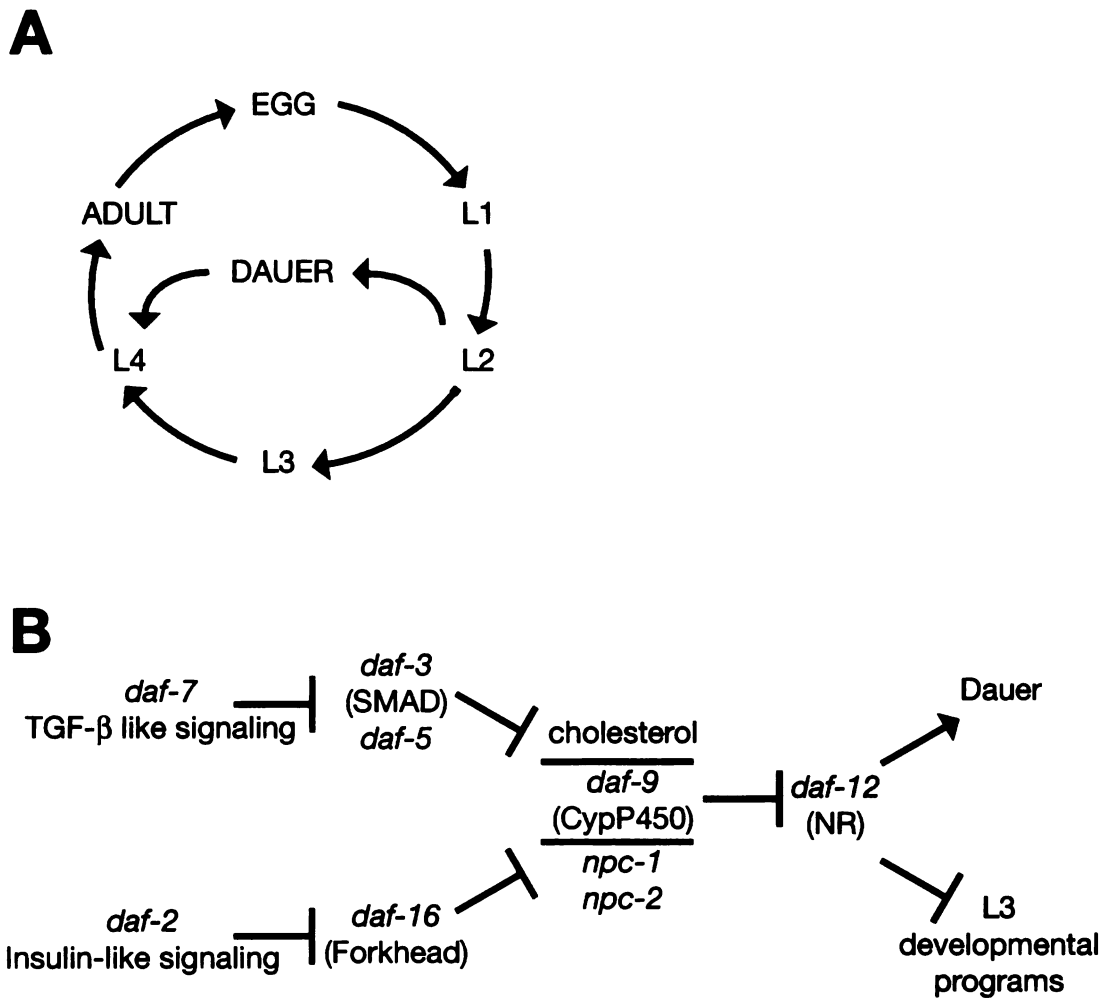


Figure 2: A putative endocrine pathway regulates dauer and gonadal migration. (A) In times of stress (the absence of food, high temperature, or overcrowding), L2 worms can enter an alternative dauer life stage. As conditions improve, dauer larva can feed and develop into adults. (B) A schematic detailing the signaling pathways leading to dauer formation. Epistasis experiments indicate that the nuclear receptor *daf-12* is downstream of both cholesterol and the *daf-9* cytochrome p450 enzyme, suggesting the possibility that *daf-12* may be regulated by a ligand synthesized from a cholesterol metabolite.

Interestingly, 10% of worms with mutations in the putative LBD of *daf*-12*(rh284)* are Daf-c. At the same time, 43% of the gonads in these *daf-12(rh284)* worms display defects in distal-tip cell migration (mig), a L3 stage specific-developmental program (Figure 6). These results suggest that a ligand might bind to the *daf*-12 LBD and regulate *daf-12* function. In this model, the *daf-12(rh284)* mutation in the *daf-12 LBD* would prevent this hormone from binding, leading to inappropriate dauer formation or the inappropriate inhibition of L3 specific developmental programs. Mutations in a cytochrome p450 enzyme, *daf-9*, also display Daf-c and mig phenotypes, and the *daf-12(rh61rh411)* null allele is downstream of daf-9 by epistasis.

In addition, a small percentage of N2 worms grown on cholesterol deficient plates have Daf-c and mig phenotypes (Gerisch *et al.*, 2001; Jia *et al.*, 2002) and again these phenotypes are placed upstream of *daf-12(rh61rh411)* by epistasis (Gerisch *et al.*, 2001). Similarly, worms lacking *npc-1* and *npc-2*, two C. elegans homologues of the human gene responsible for Nieman Pick type C disease-- a human disease characterized by altered cholesterol transport, have constitutive dauer phenotypes, and are upstream of *daf-12* by epistasis analysis (Sym *et al.*, 2000; Brown *et al.*, 2003). Placing *daf-12* or *daf-9* worms onto plates lacking cholesterol increases the penetrence of these dauer and mig phenotypes (Sym *et al.*, 2000; Gerisch *et al.*, 2001). These data suggest a pathway whereby a cytochome p450 helps synthesize a cholesterol-based ligand that regulates daf-12 function (Gerisch *et al.*, 2001; Jia *et al.*, 2002) (Figure 2B).

We sought to understand the role of cholesterol metabolites in *C. elegans.* As discussed above, these metabolites likely play roles in a wide variety of signal transduction pathways and may also serve as nuclear receptor ligands. However, cholesterol metabolism in worms has been difficult to study due to the low concentrations of cholesterol required for *C. elegans* viability (Kurzchalia and Ward, 2003). Difficulty in eliminating trace amounts of cholesterol from worm plates has led to inconsistent

phenotypes, making genetic screens impractical. Thus, we considered alternatives to growing worms on cholesterol-depleted plates. Pharmacological modification of mammalian sterol synthesis and degradation serves as the basis for many clinically important drugs, including those used as chemotherapeutics for many cancers (Nieman, 2002; Johnston and Dowsett, 2003). Therefore, we took advantage of the many drugs developed to regulate steroid hormone synthesis in mammalian cells to pharmacologically examine *C. elegans* cholesterol metabolism (Figure 3). Compounds tested include aminoglutethimide (AMG), a nonsteroidal aromatase inhibitor that blocks the conversion of cholesterol to pregnenolone, an early step in the synthesis of steroid hormones (Johnston and Dowsett, 2003); cyanoketone, an inhibitor that acts on 3 β-hydroxysteroid dehydrogenase (Sharp and Penning, 1988); equilin, an inhibitor of 17 β-hydroxysteroid dehydrogenase required for estrogen synthesis (Sawicki *et al.*, 1999); metyrapone, an inhibitor of cortisol and corticosterone synthesis by blocking 11-β-hydroxylase (Engelhardt, 1994); and ketoconazole, a general inhibitor of cytochrome p450 enzymes involved in steroid biosynthesis (Sonino, 1987). We allowed worms to grow on plates containing these drugs and looked for aberrant phenotypes similar to those observed in worms grown on plates lacking cholesterol.

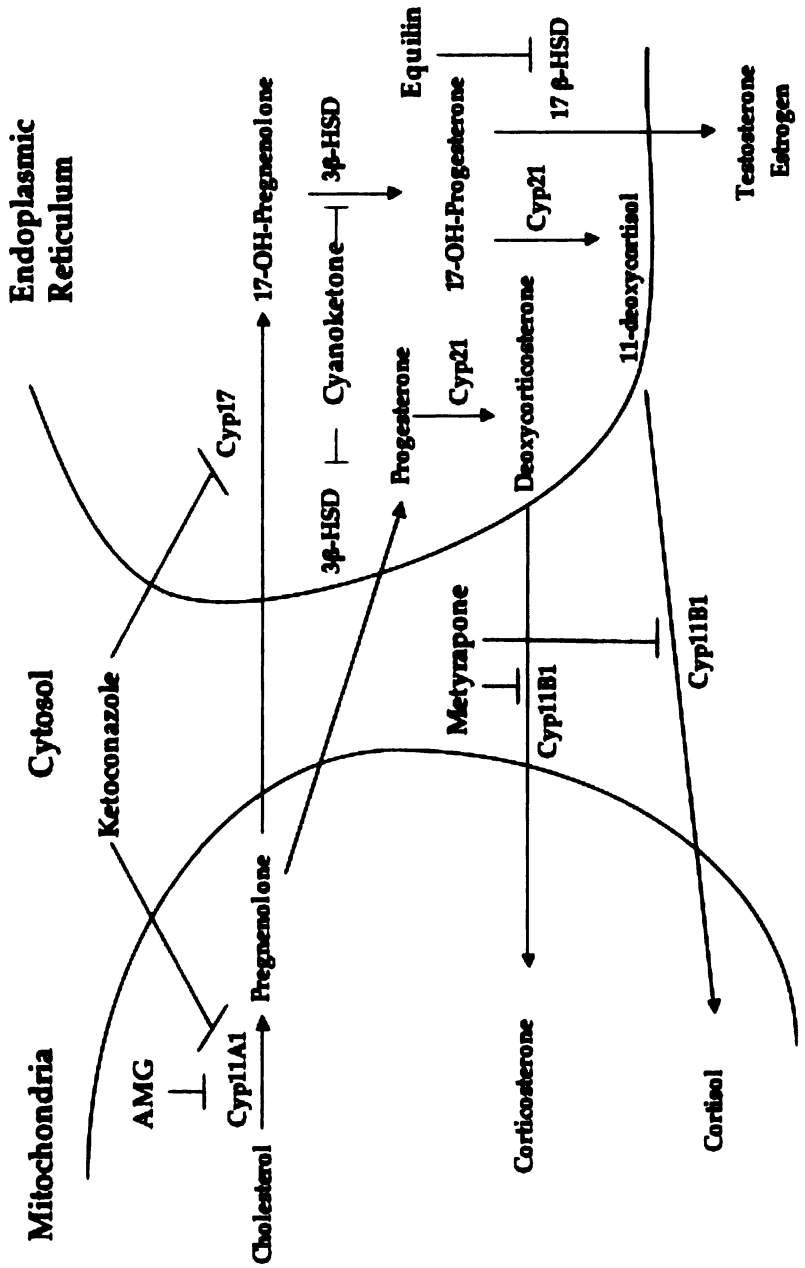


Figure 3: Mammalian steroid hormone synthesis. Diagram details the synthesis of steroid hormones from cholesterol in mammals. Pharmacological inhibitors used in this study are shown in blue. The red arrows show the enzymes inhibited by these chemical compounds.

Chapter 2: Materials and Methods

Plates, media and worm handling:

Worms were grown on NGM-lite plates containing 2 g NaCl, 4 g Bactotryptone, 3g KH_2PO4, 0.5g K_2HPO4, 20g Agar, and 8 mg cholesterol in 1 liter of media. After pouring, plates were seeded with 50 µl of OP50 bacteria, and worms were placed on the plates a day later. For experiments with chemical compounds, compounds were dissolved in 100% ethanol at 5-10 mM depending on solubility. For cholesterol depletion studies, cholesterol was left out of the plates. After autoclaving, plate media was allowed to reach 50°C, before drug was added to the text indicated concentration. As a control, in all cases, the same concentration of the ethanol solute lacking drug was added to the plates. Aberrant growth, locomotion, and reproductive behavior were observed in worms grown on plates containing above 3.5% ethanol final concentration. Therefore, all experiments were performed with plates containing alcohol concentrations below this threshold.
As mentioned by other groups (Kurzchalia and Ward, 2003; Merris *et al.*, 2003), we noticed experimental variability in worms grown on NGM-lite plates containing drugs or lacking cholesterol. Thus for some experiments, we switched to agarose (AG) plates containing agarose instead of agar, lacking Bactotryptone, and washed the OP50 bacteria 6 times before seeding plates. These plates yielded more consistent results for cholesterol depletion studies and we further observed an increased potency of drugs in these plates, even with supplemented cholesterol (Table 5). This result was presumably due to the lack of additional sterol compounds found in the Bactotryptone and the agar. These plates are referred to as AG plates throughout the manuscript.

For quantitative real time (qRT) PCR and microarray experiments, gravid adult worms were harvested from 40 HG plates (3g NaCl, 30g Agar, 20g peptone, 24 mg cholesterol, 1mM $CaCl_2$, 1 mM $MgSO_4$, and 25 mM $KP0_4$ pH 6.0 per liter).

Adult worms were bleached until 50% carcasses had been destroyed with bleaching solution: (30% Bleach (Fisher: 5.25% Sodium hypchlorite), 15 % 5N KOH in ddH_2O). Resulting eggs were washed 5x with sterile M9 buffer (5.8 g Na_2HPO_4. 3g KH_2PO_4, 0.5 g NaCl, and 1 g NH_4Cl in 1 liter), and allowed to hatch on unseeded NGM-lite plates. The next day, 40,000 worms were placed onto 15 seeded 15cm AG plates containing 50 μM ketoconazole and cholesterol as indicated. Worms were grown at 22°C. Thirty six- 40 hrs later, L4 stage worms were harvested with M9 buffer and pelleted. Pellets were resuspended in 4 mL Trizol reagent (Life Technologies) and flash frozen in liquid nitrogen.

Adult lifespan was measured by placing 60 young adult CF512 (fer-15;fem-1) (Garigan et al., 2002) worms on NGM-lite plates supplemented with 100μM of the indicated drug at 20°C. These worms do not lay eggs at restrictive temperature and thus are convenient for life span analysis. Plates were changed weekly. Worms were checked for viability every other day.

RNA preparation, microarrays, and real-time PCR analysis

To prepare RNA, worms pellets were thawed at room temperature, incubated for five min, and centrifuged for 10 min at 3,000Xg. RNA was extracted with chloroform, precipitated with isopropanol, washed with 75% EtOH, and resuspended in DEPC treated water. For microarrays, mRNA was isolated, cDNAs prepared and hybridized to arrays as previously published (Murphy *et al.*, 2003). One half of the cDNA from each condition, AG plates containing ketoconazole and/or cholesterol as noted in the text, was pooled to create a mixed reference. Equal concentrations of Cy3-labeled cDNA from each condition and the Cy5-labeled mixed reference were hybridized to an array containing all 18, 445 *C. elegans* open reading frames for 36 hours at 63°C.

Data analysis was performed with GenePix 3 and Acutiy 3.1 (Axon Instruments). The ratio of medians was calculated from the average spot intensity over the entire array and this was used to normalize the intensity of individual Cy5 and Cy3 spots. All empty, weak, streaked, over-saturated, and otherwise mutilated spots were discarded. The ratio of normalized Cy3 to Cy5 intensities was used as a measure of differential hybridization within conditions. Fold differences in hybridization were thus the log base two of this normalized value. For the purposes of the initial analysis reported here, genes with a discarded spot for any of the four conditions tested were not analyzed further. The expression ratio for each condition was then divided by the ratio found in the control worm growths, i.e. worms grown on plates containing cholesterol and lacking ketoconazole. Genes whose normalized spot intensity was greater than two fold over the controlled growth were said to be up-regulated. Genes whose normalized spot intensity was less than two fold the signal of the control growth samples were said to be down-regulated. To create Figure 7, genes with upregulated spot-intensity under both ketoconazole growth conditions (plus and minus cholesterol) relative to both growth conditions (plus and minus cholesterol) in the absence of ketoconazole were recorded.

For qRT PCR analysis, complementary DNA (cDNA) was prepared from total RNA using the RNeasy kit from Qiagen. Primers were designed to specifically amplify 100-200 nucleotides of each of the 80 *C. elegans* cytochrome p450 cDNA sequences. Primers were tested to ensure that they amplified specific products, reducing the number of tested p450 enzymes to 65. Real time PCR was performed on an Opticon 2 Continuous Fluorescence Detector DNA engine (MJ research) with Cybergreen as a fluorescent dye. Fluorescent intensity was measured after each cycle. All cycle numbers were recorded while reactions were in the linear range. Data shown is the average of two worm growths. For cycle differences, cycle numbers for each condition were subtracted from data taken from worms grown on plates containing cholesterol and lacking drug. The average

differences in cycle number between the experiments were then used to calculate the fold difference in expression, as each cycle number is equivalent to a two-fold difference in cDNA concentration. Cytochrome p450 enzymes were classified as upregulated if their expression was increased by greater than 2 fold in plates containing ketoconazole relative to plates lacking ketocoanzole in duplicate worm growths.

EMS mutagenesis

N2 L3-L4 stage worms were mutagenized in M9 buffer containing 0.5% Ethylmethanesulfonate (EMS) for four hours, worms were washed four times and pippeted onto new plates. L4-YA adult worms were then picked to new plates, and the progeny (F1) were allowed to lay eggs on ketoconazole containing plates. Isolated alleles were retested several times on drug containing plates and also tested with on plates containing other imidazole compounds as indicated. Overall, approximately 30,000 mutagenized chromosomes were tested on AG plates containing 170 μM ketoconazole, looking for suppressors.

Mapping Genes

Worms were mapped by crossing supressors to male CB4856 Hawaii strain worms. F1 worms were allowed to self-fertilize and F2 worms were selected for those that could suppress 170 μM ketoconazole. This produced cross 1 progeny. This process was repeated for each cross. After three crosses, worms were analyzed by single nucleotide polymorphism (SNP) mapping, as described in (Wicks *et al.*, 2001), to identify regions of N2 SNPs linked to the Ker phenotype. Briefly, PCR primer pairs bracketing a SNP between N2 and CB4856 in a restriction enzyme recognition site were used to amplify 250-900 base PCR products using the single worm PCR method described in (Wicks *et al.*, 2001). After PCR reactions were performed, products were digested with

Chapter 2: Results

Treatment of worms with inhibitors of mammalian sterol metabolism.

To examine the role of sterol metabolism in *C. elegans* biology, we treated worms with compounds known to inhibit mammalian sterol metabolism. Ten gravid adult N2 worms were allowed to lay eggs on NGM-lite plates containing 200 micromolar (μM) aminoglutethimide, cyanoketone, equilin, metyrapone, or ketoconazole, and the F1 progeny were examined for phenotypes. Worms developed and reproduced normally in the presence of most drugs tested, except for those grown on plates containing 200 μM ketoconazole, which exhibited delayed growth and reduced progeny (Table 1).

As shown in Table 2, F1 and F2 worms grown on NGM-lite plates containing 50 μM or higher concentrations of ketoconazole had progressively reduced progeny and growth delay. Furthermore, worms grown on 300 μM ketoconazole plates had a severe developmental delay and larval arrest. We placed L1, L2, L3, and L4 worms onto NGM-lite plates containing 250 μM ketoconazole to determine if ketoconazole could affect all developmental life stages. Indeed, ketoconazole arrested the development of L1, L2, and L3 worms, and reduced the numbers of progeny laid by L4 worms, suggesting it blocks a signaling pathway required at all worm stages and/or multiple physiological pathways in *C. elegans* (Table 3). Interestingly, while ketoconazole affected the number of eggs laid by the worms, and the development of hatched worms, it did not seem to affect worm embryogenesis or egg hatching per se.

The effects of ketoconazole on worm development and reproduction were similar to the phenotypes observed in worms grown on cholesterol deficient medium (Table 2 and (Yochem *et al.*, 1999; Shim *et al.*, 2002; Merris *et al.*, 2003). Thus, we tested if cholesterol depletion could synergize with ketoconazole. Removal of cholesterol from the medium potentiated the effects of ketoconazole (Table 2). For example, whereas

Drug	Growth	Brood Size
200 μM Aminoglutethimide (AMG)	No effect	No effect
200 μM Cyanoketone	No effect	No effect
200 μM Equilin	No effect	No effect
200 μM Metyrapone	No effect	No effect
200 μM Ketoconazole	Delayed	Severely reduced

Table 1: Ketoconazole causes a severe growth and egg laying defect. Adult N2 worms were allowed to lay eggs on NGM-lite plates supplemented with cholesterol and indicated drugs. F1 worms were then observed for phenotypes.

Ketoconazole concentration	Cholesterol	Brood Size/worm F1	F2	Growth effect
-	+	254	298	-
-	-	178	39	F2 growth delayed
50 μM	+	188	99	F2 growth delayed
50 μM	-	104	23	F2 growth delayed
100 μM	+	134	51	F1 growth slightly delayed
100 μM	-	91	13	F1 growth slightly delayed
200 μM	+	43	0	F1 growth delayed
200 μM	-	10	0	F1 growth delayed and some larval arrest
300 μM	+	0	0	F1 L1 or L2 arrest

Table 2: Cholesterol deprivation potentiates the effect of Ketoconazole. Adult N2 worms were allowed to lay eggs on NGM-lite plates supplemented with cholesterol or ketoconazole as indicated. F1 worms were then observed for phenotypes.

stage	Growth effect		
	Day 1	Day 2	Day 3
Egg	Hatched, L1	L1	L1
L1	L1	L1/arrest	L1/L2/arrest
L2	L2	L2/L3/arrest	L2/L3/arrest
L3	L3	L3/arrest	L3/L4
L4	L4/young adult	adult (has less eggs than normal worms)	adult (has less eggs than normal worms)

Table 3: Ketoconazole affects all worm life stages. N2 worms of indicated stage were placed on NGM-lite plates containing 250 μM ketoconazole and observed for phenotypes.

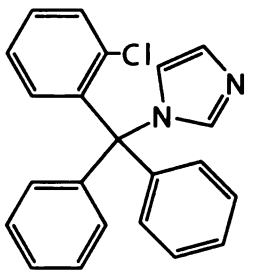
200 μM ketoconazole NGM-lite plates supplemented with 8 mg/ml cholesterol led to a mild growth defect, worms grown on 200 μM ketoconazole NGM-lite plates lacking supplemented cholesterol displayed a severe growth delay and a small percentage of worms arrested during development.

Ketoconazole is from a family of p450 inhibitors

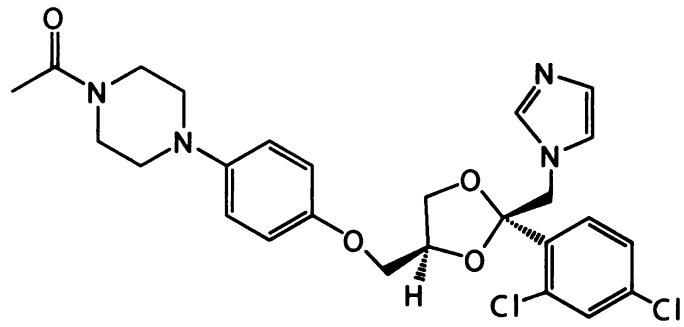
Ketoconazole belongs to a family of imidazole based compounds (including clotrimazole, sulconazole, and miconazole) (Figure 4) that block a wide spectrum of cytochrome p450 enzymes in multiple organisms (Sonino, 1987; Urbina *et al.*, 1995; Zhang *et al.*, 2002) and have been used clinically to treat fungal infections and cancers (Sonino, 1987). Members of this family block cytochrome p450 enzymes by binding to the active site of the enzyme. Interestingly, these compounds block overlapping yet distinct classes of cytochome p450 enzymes. In fact, sulconazole and miconazole are thought to block a larger number of p450 enzymes with greater potency than ketoconazole (Zhang *et al.*, 2002). To determine if these additional compounds might affect worm physiology, we placed adult worms (P0) on plates containing clotrimazole, sulconazole, and miconazole and looked for phenotypes in F1 progeny. As shown in Table 4, clotrimazole, sulconazole, and miconazole had much stronger effects on worm growth and reproduction than ketoconazole, consistent with their increased potency and promiscuity for mammalian p450s (Zhang *et al.*, 2002). These results suggest that imidazole-based compounds may be blocking *C. elegans* p450 enzymes *in vivo*.

Imidazole based inhibitors reduce worm lifespan

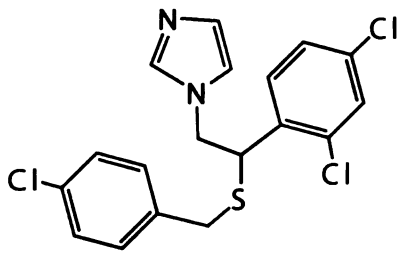
As cholesterol metabolism has been suggested to play a role in worm lifespan (Tatar *et al.*, 2003), we examined the effect of these drugs on worm lifespan. Sixty young adult CF512 worms were placed onto NGM-lite plates containing 100 μM of the



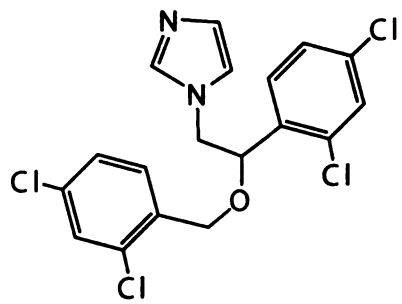
CLOTRIMAZOLE



KETOCONAZOLE



SULCONAZOLE



MICONAZOLE

Figure 4: Imidazole family compounds used in this study. Chemical structures of different compounds used are shown.

Type of plate	Day 2	Day 3	Day 4
NGM-lite	L3, L4	A, E, L1	A, E, L1, L2
Carrier (EtOH)	L3, L4	A, E	A, E, L1, L2
100 μM ketoconazole	L3, L4	A, a few eggs	A, E, L1
100 μM sulconazole	L1, dead	L1, L2, dead	L2, dead
100 μM clotrimazole	L1, L2	L2, L3 dead	all dead
100 μM miconazole	L1	L2/dead	L2/dead

Table 4: Ketoconazole related p450 inhibitors have strong effects on worm growth and reproduction. Adult N2 worms were allowed to lay eggs on NGM-lite plates supplemented with cholesterol and indicated drugs.

indicated drug. These worms are sterile at restrictive temperature, facilitating ease of analysis. AMG, equlin, metyrapone and cyanoketone had no effect on worm lifespan. However, ketoconazole, miconazole, clotrimazole, and sulconazole slightly reduced worm lifespan (Figure 5).

Npc-1 worms are sensitive to ketoconazole

We also tested for a functional interaction between ketoconazole and *npc1* mutant worms. These worms have a deletion in one of the worm homologues of the human gene responsible for Niemann-Pick type C disease, a protein thought to be involved in cholesterol transport. *Npc-1* worms are more sensitive to cholesterol depletion than N2 worms, but have no other published phenotypes (Sym *et al.*, 2000). To determine if ketoconazole might functionally interact with *npc-1*, we placed adult *npc-1* worms on NGM-lite plates containing 50 μM ketoconazole, and looked for phenotypes in the F1 progeny. Interestingly, we observed the mig phenotype (Figure 6) in *npc-1* worms, but not in N2 worms grown on these plates (Table 6). Mig phenotypes have previously been identified in worms containing mutations in *daf-12* (a nuclear receptor) and *daf-9* (a cytochrome p450). We noticed that drugs were more potent in AG plates than in NGM-lite plates (Table 5), presumably because AG plates have less sterols than NGM-lite plates due to the presence of agarose rather than agar, lack of bactotryptone, and use of washed bacteria. Therefore, we also tested *npc-1* worms on AG plates containing ketoconazole. As previously reported, *npc-1* worms were more sensitive to cholesterol depletion than N2 worms (Table 8). In addition, *npc-1* worms were more sensitive to ketoconazole than N2 worms: on 50 μM ketoconazole AG plates, *npc-1* worms arrested during larval development, while N2 worms did not (Table 8). In summary, *npc-1* worms are more sensitive to cholesterol depletion and ketoconazole, than N2 worms.

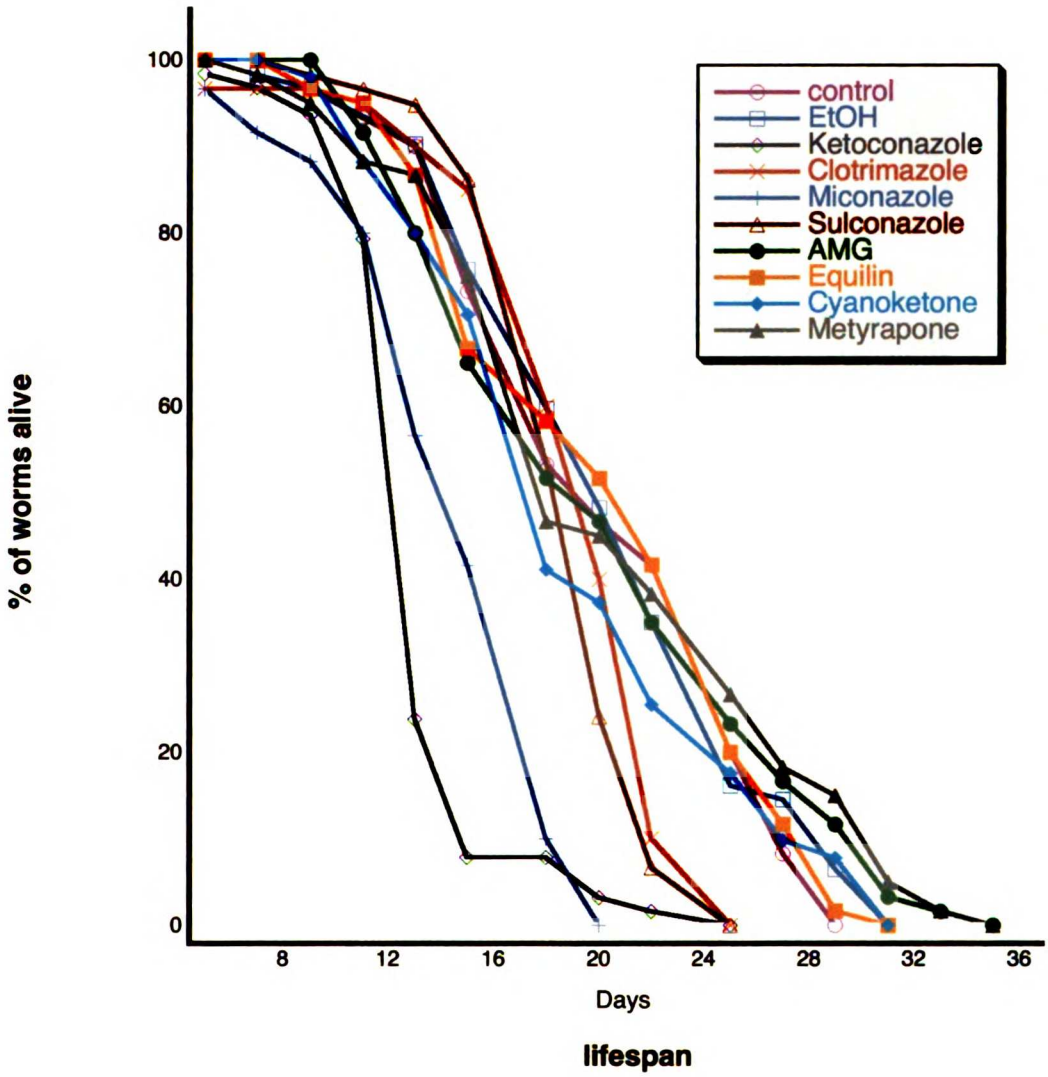


Figure 5: Imidazole drugs reduce worm lifespan. Adult lifespan was measured by placing 60 young adult CF512 (fer-15;fem-1) (Garigan et al., 2002) worms on NGM-lite plates supplemented with 100 μM of the indicated drug at 20°C. These worms do not lay eggs at restrictive temperature and thus are appropriate for life span analysis. Plates were changed weekly. Worms were checked for viability every other day.

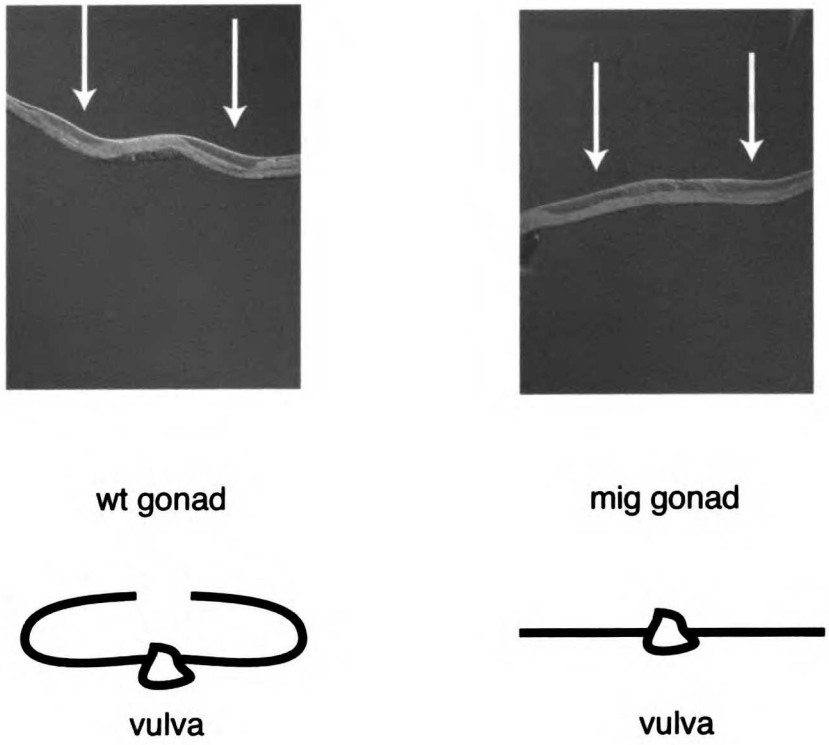


Figure 6: Worms with mig phenotypes. Panels display N2 worms with gonadal migration phenotypes at 25x magnification and a diagram displaying the altered migration in cartoon form. Pictures are typical of gonadal migration defects recorded in Tables 6 and 7.

Plate type	Ketoconazole	Cholesterol	Brood Size	Growth
NGM-lite	0 μM	+	254	no effect
	50 μM	+	188	no effect
	100 μM	+	134	slight
	200 μM	+	43	delayed
AG	0 μM	+	240	no effect
	50 μM	+	100	very slight
	100 μM	+	0	delayed
	200 μM	+	0	larval arrest

Table 5: Ketoconazole is more potent inhibitor of worms in AG plates than in NGM-lite plates. Adult N2 worms were allowed to lay eggs on NGM-lite or AG plates supplemented with cholesterol and ketoconazole at indicated concentration. F1 worms were observed for growth and reproductive phenotypes.

Worm Strain	Plates	% mig worms
N2 worms	NGM-lite + cholesterol	0% (250)
	NGM-lite - cholesterol	0% (100)
	50 μM Ketoconazole + cholesterol	0% (100)
npc-1	NGM-lite + cholesterol	0% (100)
	NGM-lite - cholesterol	0% (50)
	50 μM Ketoconazole + cholesterol	45% (70)

Table 6: Npc-1 worms have gonadal migration (mig) defects on plates containing a low concentration of ketoconazole. Adult N2 or *npc-1* worms were allowed to lay eggs on NGM-lite plates supplemented with cholesterol and/or ketoconazole as indicated. F1 worms were then observed for phenotypes.

Role of Ketoconazole in the Dauer and gonadal migration pathway.

The mig phenotype observed in npc-1 worms grown on NGM-lite ketoconazole plates, suggested a genetic interaction between cholesterol and ketoconazole leading to the mig phenotype. Therefore, we looked at N2 worms grown on the ketoconazole AG plates, reasoning that such N2 worms might have gonadal migration defects yet not growth defects since the plates were supplemented with 8 μg/ml cholesterol. Indeed, 24% of worms grown on AG plates containing cholesterol and 50 μM ketoconazole had gonadal migration defects (Figure 6 and Table 7); a similar result was observed on plates lacking cholesterol (Table 7) (Gerisch *et al.*, 2001).

The mig defect in worms grown on cholesterol-free plates is suppressed by *daf-12* null alleles (*rh61rh411*) (Gerisch *et al.*, 2001). Therefore, we investigated the phenotype of *daf-12* worms on AG plates containing cholesterol and 50 μM ketoconazole. *daf-12(rh61rh411)* worms grown on these plates did not display the mig phenotype, suggesting that ketoconazole affects proteins upstream of *daf-12* (Table 7).

We also asked if *daf-12(rh61rh411)* could suppress all ketoconazole induced phenotypes or just the defect in gonadal migration. *Daf-12(rh61rh411)* worms did not suppress the larval arrest observed when worms were grown on 250 μM ketoconazole (Table 8). Furthermore, *daf-12(rh61rh411)* and N2 worms displayed a similar growth delay on plates containing 100 μM ketoconazole. Similarly, *daf-12(rh61rh411)* worms failed suppress the larval arrest phenotype observed when worms were grown on cholesterol deficient plates (Table 8). Thus, *daf-12(rh61rh411)* could rescue only a subset of ketoconazole and cholesterol depletion induced phenotypes, suggesting that ketoconazole regulates multiple signaling molecules in addition to *daf-12*.

Worms upregulate p450 mRNA expression in response to ketoconazole.

Since our experiments suggested that ketoconazole regulates multiple pathways

Worm Strain	Plates	% mig worms
N2 worms	plates + cholesterol	0% (250)
	plates - cholesterol	10% (100)
	50 μM Ketoconazole + cholesterol	24% (273)
rh61rh411 (daf-12)	plates + cholesterol	0% (140)
	plates - cholesterol	0% (100)
	50 μM Ketoconazole + cholesterol	2% (276)

Table 7: N2 worms have a mig phenotype on ketoconazole NG-agarose plates. Adult N2 or daf-12 null (r61rh411) worms were allowed to lay eggs on AG plates supplemented with cholesterol and/or ketoconazole as indicated. F1 worms were then observed for phenotypes.

Worm Strain	Plates	% L4 or older	
		F1	F2
N2 worms	AG plates + cholesterol	100% (100)	100% (100)
	AG plates - cholesterol	100% (100)	2% (100)
	50 μM Ketoconazole + cholesterol	100% (100)	90% (100)
	200 μM Ketoconazole + cholesterol	4% (100)	0%
npc-1	AG plates + cholesterol	100%	100%
	AG plates - cholesterol	28% (100)	0%
	50 μM Ketoconazole + cholesterol	12% (100)	0%
	200 μM Ketoconazole + cholesterol	0% (100)	0%
rh61rh41 (daf-12)	AG plates + cholesterol	100% (100)	100% (100)
	AG plates - cholesterol	100% (100)	1% (100)
	50 μM Ketoconazole + cholesterol	100%	92% (100)
	200 μM Ketoconazole + cholesterol	8% (100)	0%

Table 8: Growth effects of ketoconazole and cholesterol depletion on the growth and development of N2, *npc-1,* and *daf-12* (rh61rh411) worms. Adult N2, *daf-12* null (r61rh411), or *npc-1* worms were allowed to lay eggs on AG plates supplemented with cholesterol or ketoconazole as indicated. F1 and F2 worms were then observed for phenotypes.

in *C. elegans*, we performed microarray analysis to examine gene expression changes in response to ketoconazole. Starved L1 worms were plated on AG plates that contained or lacked cholesterol and 50 μM ketoconazole, a concentration that did not affect growth. Worms were allowed to grow to the L4 stage before harvest and used to isolate mRNA and synthesize cDNA. cDNA was hybridized to microarrays covering all of the 18,455 predicted *C. elegans* open reading frames. All conditions were compared to a mixed reference of cDNA taken from each experimental condition. Data shown are the preliminary results of a single experiment. However, upregulated genes shown in Figure 7 had increased expression in worms grown on plates containing ketoconazole relative to worms grown on plates lacking ketoconazole in two separate worm growths, i.e. both in the presence and absence of cholesterol. Upregulated genes include six cytochrome p450 enzymes (Figure 7A), putative deaminases and carboxylases and other genes that might play a role in regulating small molecule metabolism (Figure 7B), proteins likely involved in cell signaling (Figure 7C), and many proteins of unknown function (Figure 10).

The upregulation of p450 enzymes observed on the microarrays are consistent with observations in mammalian cells, where treatment with ketoconazole has been shown to increase p450 expression, presumably through a compensation mechanism (Suzuki *et al.*, 2000). To confirm the accuracy of the microarray results and determine if ketoconazole affects other cytochrome p450 expression, we analyzed the mRNA expression of 67 *C. elegans* p450 enzymes by qRT PCR. The presence of ketoconazole caused a significant increase in the expression of thirty of the cytochrome p450 enzymes, suggesting that ketoconazole might inhibit worm p450 function. Some p450 enzymes like Cyp14A4, Cyp33C2, and Cyp34A7 were upregulated more than 500 fold (Figure 8A). The six p450 enzymes observed to be upregulated using the microarrays were among those upregulated by qRT PCR as well (Figure 8A). These results are consistent with the hypothesis that ketoconazole alters p450 enzyme activity in worms, leading to an altered

A

Gene	---	Chol	Ket	Ket+Chol	Gene function:
K07C6.3	1.8	1.0	36.4	39.0	cyp450
R04D3.1	1.4	1.0	23.4	35.2	cyp450
R08F11.3	1.4	1.0	7.4	4.2	cyp450
K09D9.2	0.3	1.0	4.4	4.6	cyp450
C49G7.8	0.4	1.0	4.0	5.5	cyp450
C36A4.3	1.0	1.0	3.0	4.1	cyp450
B0213.15	0.9	1.0	2.0	2.1	cyp450

B

Gene	---	Chol	Ket	Ket+Chol	Gene function:
R05D8.c	4.1	1.0	23.3	24.2	alcohol/ribitol dehydrogenase
C47A10.1	0.2	1.0	14.1	15.8	ATP-binding cassette (ABC) transporter activity
W01A11.1	0.6	1.0	7.0	6.9	Epoxide hydrolase activity [P]
F46E10.4	0.5	1.0	6.0	6.0	epoxide hydrolase
K07E3.8	0.9	1.0	4.4	5.0	vem-1; heme-binding domain
H10D18.6	1.1	1.0	4.3	3.9	Member of the UDP-glucoronosyl transferase family
F15B9.1	1.3	1.0	4.2	4.5	choloylglycine hydrolase or ASAH acid ceramidase
W01A11.1	0.6	1.0	7.0	6.9	Epoxide hydrolase activity [P]
F46E10.4	0.5	1.0	6.0	6.0	epoxide hydrolase
C47G2.1	1.1	1.0	5.2	3.4	(cut1) Extracellular matrix structural constituent
K07E3.8	0.9	1.0	4.4	5.0	vem-1; heme-binding domain
H10D18.6	1.1	1.0	4.3	3.9	Member of the UDP-glucoronosyl transferase family
C34C6.7	1.0	1.0	4.3	3.8	related to map1b, binds microtubules and phospholipids
F15B9.1	1.3	1.0	4.2	4.5	choloylglycine hydrolase or ASAH acid ceramidase

C

Gene	---	Chol	Ket	Ket+Chol	Gene function:
C05D9.8	1.4	1.0	4.9	3.6	von Willebrand factor type A domain family
T03F7.7	0.6	1.0	3.6	2.4	CRAL-TRIO domain, transcription factor
F33E11.4	1.1	1.0	3.3	2.4	Transcription factor
C01B10.6	1.0	1.0	2.6	2.5	notch 3 homologue
F42C5.10	0.6	1.0	2.5	2.1	osmoregulation and growth regulation
F08E10.6	1.1	1.0	2.1	2.6	Nematode 7TM chemoreceptor GPCR
F13H6.1	1.3	1.0	2.9	1.9	high similarity to GCP-1

Figure 7: Preliminary microarray analysis of worms grown on ketoconazole containing media. 40,000 worms were grown on AG plates supplemented with cholesterol and/or 50 μM ketoconazole as indicated. RNA was harvested using Trizol and cDNA was prepared. CDNA was labeled with Cy5 or Cy3 dyes and hybridized to an array containing all 18, 445 *C. elegans* open reading frames. ORFs with greater than two fold increased expression in N2 worms grown on ketoconazole containing plates (Ket and Ket+Chol) relative to the expression observed in worms grown on plates lacking ketoconazole (--- and Chol). Pale yellow: less than two fold change. Bright yellow: 2 fold – 5 fold change. Orange: 5 fold – 20 fold change. Red: 20 fold – 100 fold change. These genes can be summarized into three groups: cytochrome p450 genes (A), genes likely involved in small molecule metabolism (B), genes involved in signaling pathways (C), and genes of unknown function (Figure 10).

A

Cyp	Gene	---	Chol	Ket	Ket+Chol	
13A2	T10B9.7	1.2	1.0	4.8	5.2	
13A3	T10B9.5	2.4	1.0	15.8	24.0	
13A6	T10B9.3	1.6	1.0	7.0	6.3	
13A8	T10B9.4	3.1	1.0	11.6	8.7	
13A10	ZK1320.4	0.9	1.0	9.5	10.5	
14A4	R04D3.1	0.9	1.0	3716.2	4031.5	***
25A1	C36A4.1	1.1	1.0	2.6	5.2	
25A2	C36A4.2	0.9	1.0	3.4	5.8	
25A3	C36A4.3	1.1	1.0	12.7	20.1	***
29A2	T19B10.1	1.0	1.0	6.0	8.3	
29A3	Y38C9B.1	0.9	1.0	6.7	8.4	
33B1	C25E10.2	1.1	1.0	4.5	3.0	
33C1	C45H4.2	1.5	1.0	459.8	483.8	
33C2	C45H4.17	1.5	1.0	819.5	906.4	
33C4	F44C8.1	1.5	1.0	4.7	6.1	
33C6	F41B5.7	0.5	1.0	17.2	21.9	
33C7	F41B5.2	1.1	1.0	28.0	31.8	
33C8	R08F11.3	1.3	1.0	5.7	12.9	***
33D1	K05D4.4	1.3	1.0	18.2	23.9	
33D3	Y17D7A.4	1.5	1.0	23.4	24.1	
34A5	B0213.10	1.3	1.0	9.0	9.7	
34A6	B0213.11	1.0	1.0	43.7	69.7	
34A7	B0213.12	1.2	1.0	831.0	1088.3	
34A8	B0213.14	1.0	1.0	33.6	35.5	
34A9	B0213.15	1.0	1.0	5.0	6.7	***
35A1	C03G6.14	1.5	1.0	10.8	13.7	
35A3	K09D9.2	1.0	1.0	6.1	12.0	***
35A4	C49G7.8	1.2	1.0	11.7	22.0	***
35B1	K07C6.4	1.4	1.0	13.3	21.1	
35B2	K07C6.3	1.5	1.0	335.6	322.9	***
35B3	K07C6.2	1.7	1.0	62.2	110.1	

B

Cyp	Cosmid	No difference	Upregulated
13A1	T10B9.8	***	
13A2	T10B9.7		***
13A3	T10B9.5		***
13A4	T10B9.1	***	
13A5	T10B9.2	***	
13A6	T10B9.3		***
13A7	T10B9.10	***	
13A8	T10B9.4		***
13A9	T10B9.6	***	
13A10	ZK1320.4		***
13A11	F14F7.2	***	
13A12	F14F7.3	***	

Figure 8: Worms grown on ketocoanzole have substantially increased p450 expression. (A) Worm cytochome p450 expression was measured by quantitative real-time PCR. 40,000 worms were grown on AG plates supplemented with cholesterol and/or 50μM ketoconazole as indicated. RNA was harvested using Trizol and cDNA was prepared. Primers were used to amplify 67 of the 80 estimated worm p450 enzymes and expression of each P450 was compared between conditions. Fold differences are expressed relative to plates containing EtOH and cholesterol. Each worm growth was performed twice. Pale yellow: less than two fold change. Bright yellow: 2 fold – 5 fold change. Orange: 5 fold – 20 fold change. Red: 20 fold – 100 fold change. Brown: Greater than 100 fold change. Genes noted with "***" were also upregulated on the microarrays (Figure 7). (B) Table showing that not all Cyp13 family members are upregulated, even though many are on the same cosmid.

spectrum of small molecules. Cholesterol deprivation alone had only a mild effect on p450 expression, with only two p450 enzymes, Cyp13A3 and Cyp13A8, upregulated 2.4 and 3.1 fold, respectively (Figure 8).

A genetic screen to identify genetic supressors of ketoconazole.

As high concentrations of imidazole drugs induce larval arrest, we used ketoconazole as the basis for a genetic screen. We EMS-mutagenized 30,000 genomes of N2 worms in several pilot screens, and plated F2 mutant worms on NGM-lite plates containing 250 μM ketoconazole. We identified 15 ketoconazole resistant strains (Ker) and studied two of them (Ker-6 and Ker-12) in further detail. Neither Ker-6 nor Ker-12 strains were able to suppress the larval arrest induced by sulconazole, clotrimazole, or miconazole (Table 9). Indeed, Ker-6 was more sensitive to these drugs than N2 worms, whereas Ker-12 was able to suppress lower concentrations of these drugs (Table 9).

As Ker-12 was the most robust suppressor of ketonconazole induced larvarl arrest, we mapped the gene responsible Ker-12using the SNP mapping approach (Wicks *et al.*, 2001). Ker-12 worms were crossed with CB4856 male worms, having numerous single nucleotide polymorphisms (SNP) relative to the N2 worm strain (isolated in Bristol, England). F2 worms were tested for growth on plates containing 250 μM ketoconazoleand then crossed once more with CB4856 male worms. After three generations of crossing, a set of PCR primers specific for the two arms and center of each of the six worm chromosome, were used to identify a genomic region in suppressor worms that remained linked to N2 SNPs (Figure 9). Single worm PCR was used to amplify the DNA of Ker-12 mutants that had been crossed three times with CB4856. Amplified fragments were then tested for the presence of the N2 or CB4856 SNP pattern at each locus. Using this approach, the region containing the Ker-12 mutation was narrowed to two possible regions, an 8.6 MB segment between markers c16c8 and

Drug Used	Strain	Day 2	Day 3	Day 4
Ngm-lite	N2	L2, L3 , L4	L3, YA, A, E	A , E, L1, L2
	Ker6	L2, L3, L4	A, E, L1	A , E, L1, L2
	Ker12	L3,L4	A, E, L1	A , E, L1, L2
250μM ketoconazole	N2	L1 , L2	L1, L2	L1, L2
	Ker6	L2, L3	L2, L3	L2, L3, L4
	Ker12	L2, L3	L3, L4	L3, L4
125μM miconazole	N2	L1, dead	L1, L2, dead	L1, L2, dead
	Ker6	dead	dead	dead
	Ker12	L2, dead	L2, L3, dead	L2-L4, dead
125μM sulconazole	N2	L1, L2, dead	L2, dead	L2, dead
	Ker6	L1, dead	dead	dead
	Ker12	L1, L2	L2, dead	L2, dead
250μM clotrimazole	N2	L1, dead	L1, L2, dead	dead
	Ker6	L1,L2, dead	dead	dead
	Ker12	L1, L2, dead	L2 , dead	L2 , dead

Table 9: Worm strains that suppress ketoconazole effect do not suppress other imidazole compounds. Indicated worm strains were allowed to lay eggs on NGM-lite plates supplemented with cholesterol and indicated drugs and F1 worms were observed for phenotypes.

A

Chromosome	Lane	Locus	Clone	Enzyme	N2 pattern	CB4856 pattern	Ker-12	Ker-12 cross 3
I	1	0.7 MB	Y18H1A	DraI	241,285	526	N2	CB4856
I	2	4.6 MB	B0041	DraI	238,107,103	154,107,103,84	N2	CB4856
I	3	14 MB	C37A5	EcoRI	253,149	402	N2	CB4856
II	4	0.1 MB	T01D1	DraI	402,172,44	574,44	N2	CB4856
II	5	6.8 MB	T13C2	DraI	299,125,70	369,125	N2	N2
II	6	13.2 MB	F15D4	DraI	516	396,120	N2	CB4856
III	7	0.8 MB	K02F3	MseI	184,123	184,60,63	N2	CB4856
III	8	7 MB	F31E3	AvaII	300,198	489	N2	CB4856
III	9	12.5 MB	Y111B2A	MseI	521	273,248	N2	CB4856
IV	10	1.5 MB	K03H6	HpaII	244,213	457	N2	CB4856
IV	11	5 MB	E03H12	DraI	455,71,57	283,172,7	N2	CB4856
IV	12	16 MB	Y105C5B	DraI	189,78,37	267,37	N2	CB4856
V	13	1.8 MB	F36H9	DraI	332,82,79	411,82	N2	CB4856
V	14	7 MB	VC5	DraI	432,79	297,135,7	N2	CB4856
V	15	18 MB	Y51A2D	DraI	393	274,119	N2	CB4856
X	16	1.3 MB	C52B11	MseI	239,55,49	294,49	N2	CB4856
X	17	8 MB	F45E1	EcoRI	516,259	776	N2	N2
X	18	14 MB	R03E1	MseI	250	200,50	N2	N2

B

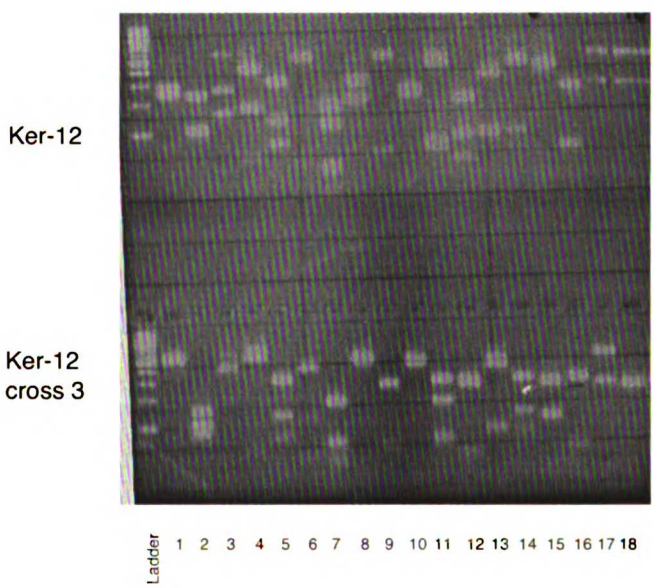


Figure 9: SNP mapping of genomic loci responsible for Ker-12 phenotype. (A) Table of SNP markers, restriction enzymes, digestion patterns, and summary results of initial SNP mapping. These markers span each of the six worm chromosomes, a SNP for each arm and the center of each chromosome. (B) DNA fragments containing the SNP were amplified from worm genomic DNA. Amplified fragments were digested with indicated restriction enzymes and resolved on a 3% agarose gel. Top: pattern from Ker 12 worms. Bottom: pattern from Ker-12 worms that had been crossed three times with CB4856 males and tested after each cross to ensure that the Ker phenotype was maintained. Region containing Ker-12 mutation should be linked to N2 SNP markers.

Gene	---	Chol	Ket	Ket+Chol	Gene function:
E02C12.6	0.98	1.00	44.44	48.07	unknown duff protein 227
E02C12.11	1.07	1.00	20.62	37.82	unknown duff protein 227
E02C12.10	1.34	1.00	17.24	20.90	unknown duff protein 227
E02C12.8	0.80	1.00	11.50	18.00	unknown duff protein 227
F48G7.12	0.68	1.00	11.19	12.28	unknown duff protein 227
F58B4.5	0.22	1.00	10.75	10.80	unknown duff protein 227
Y46C8_95.a	1.22	1.00	10.19	5.08	unknown
C34H4.2	1.28	1.00	8.77	5.78	unknown duff 274 family
Y46C8_103.a	0.97	1.00	4.03	6.96	unknown
T16G1.4	0.92	1.00	3.93	5.05	unknown duff 227
C49G7.10	1.19	1.00	3.76	3.22	unknown
ZK896.2	1.10	1.00	3.43	3.19	unknown
C05D2.8	1.29	1.00	3.20	2.97	unknown
Y67D8A_360.a	0.55	1.00	2.72	2.22	unknown
K08D8.6	1.21	1.00	2.63	2.35	unknown
Y108G3B.a	1.26	1.00	2.44	1.80	unknown
C29F7.2	0.84	1.00	2.23	2.80	unknown
Y87G2A.p	0.83	1.00	2.23	1.60	unknown
Y15E3B.f	0.82	1.00	2.20	1.83	unknown

Figure 10: Upregulated genes in microarray experiments of unknown function. 40,000 worms were grown on NGM-agarose plates supplemented with cholesterol and/ or 50μM ketoconazole as indicated. RNA was harvested using Trizol and cDNA was prepared. CDNA was labeled with Cy5 or Cy3 dyes and hybridized to an array containing all 18, 445 *C. elegans* open reading frames. ORFs with greater than two fold increased expression in N2 worms grown on ketoconazole containing plates (Ket and Ket+Chol) relative to the expression observed in worms grown on plates lacking ketoconazole (--- and Chol). Pale yellow: less than two fold change. Bright yellow: 2 fold – 5 fold change. Orange: 5 fold – 20 fold change. Red: 20 fold – 100 fold change. Duff proteins are proteins with computer defined homologous domains of unknown function.

Cyp	Gene	---	Chol	Ket	Ket+Chol
13A1	T10B9.8	1.96	1.00	1.56	2.17
13A4	T10B9.1	1.48	1.00	1.99	1.96
13A5	T10B9.2	1.44	1.00	4.09	5.09
13A7	T10B9.10	1.31	1.00	1.36	1.37
13A9	T10B9.6	1.59	1.00	1.35	1.36
13A11	F14F7.2	1.62	1.00	1.72	1.62
13A12	F14F7.3	1.07	1.00	2.27	1.93
13B1	F02C12.5a	0.71	1.00	0.58	0.91
13B2	K06G5.2	1.01	1.00	0.66	0.99
14A1	K09A11.2	1.68	1.00	1.24	1.69
14A3	K09A11.4	1.05	1.00	1.14	1.93
14A5	F08F3.7	1.01	1.00	0.86	1.19
22A	T13C5.1	1.18	1.00	1.48	2.23
25A4	C36A4.6	1.04	1.00	0.72	1.25
25A6	K06B9.1	1.00	1.00	1.00	0.79
29A1	C44C10.2	0.94	1.00	0.53	1.24
23A	B0331.1	1.00	1.00	1.89	1.56
31A1	C01F6.3	1.18	1.00	1.08	1.44
31A2	H02I12.8	0.77	1.00	0.35	0.42
31A3	Y17G9B.3	1.04	1.00	0.69	0.60
32A	C26F1.2	1.40	1.00	1.54	1.47
33A1	C12D5.7	1.20	1.00	1.61	2.11
33C3	F41B5.4	1.31	1.00	0.96	0.95
33C9	C50H11.15	1.74	1.00	2.90	6.12
33C10	F41B5.5	1.61	1.00	0.94	1.23
33C11	Y49C4A.9	2.94	1.00	3.55	3.99
33E2	F42A9.5	1.03	1.00	2.56	3.43
34A1	F42A9.4	1.44	1.00	2.66	3.41
34A2	T10H4.11	1.45	1.00	0.99	1.37
34A3	C41G6.1	3.12	1.00	1.56	2.87
34A4	T09H2.1	1.49	1.00	1.21	1.01
34A10	B0213.16	1.06	1.00	1.35	1.52
35A2	C03G6.15	2.91	1.00	0.30	1.45
35A5	K07C6.5	1.00	1.00	0.81	1.06
37A1	F01D5.9	1.30	1.00	1.68	2.71
37B1	F28G4.1	1.53	1.00	1.28	3.00
42A	Y80D3A.5	0.90	1.00	0.72	1.24
43A	E03E2.1	1.01	1.00	1.09	1.05

Figure 11: p450 enzymes that are not upregulated in worms grown on ketoconazole. Worm cytochome p450 expression was measured by quantitative real-time PCR. 40,000 worms were grown on AG plates supplemented with cholesterol and/or 50μM ketoconazole as indicated. RNA was harvested using Trizol and cDNA was prepared. Primers were used to amplify 67 of the 80 estimated worm p450 enzymes and expression of each P450 was compared between conditions. Fold differences are expressed relative to plates containing EtOH and cholesterol. Each worm growth was performed twice. Genes with upregulated expression in only one of the two worm growths were classified as "not upregulated."

Chapter 2: Discussion

Cholesterol is required for many aspects of *C. elegans* biology. In the absence of cholesterol, worms display defects in growth, reproduction, gonadal migration, dauer formation, and molting. However, the specific role of cholesterol in these pathways is not established. As cholesterol serves as the precursor of hormones and signaling molecules in many organisms, we treated worms with a panel of inhibitors of mammalian steroid hormone biosynthesis and looked for phenotypes. Most of the drugs tested had no effect on the worms. This could be due to a failure of the drug to inhibit a biologically important enzyme, or even to enter the worms. Alternatively, the worms could quickly metabolize the drugs, or pump out drug after entry. For example, the worm homologue of 17β-HSD, *let-767*, had growth and reproductive phenotypes, yet an inhibitor of mammalian 17β-HSD (equilin) had no effect on worms (Table 1). In contrast, a class of imidazole-based steroid hormone synthesis inhibitors affected growth, reproduction, gonadal migration, and lifespan defects. These drugs have previously been shown to bind the catalytic site of several classes of cytochrome p450 enzymes and block their activity in multiple organisms (Sonino, 1987; Urbina *et al.*, 1995; Zhang *et al.*, 2002). *C. elegans* p450 enzymes are most closely related to their mammalian counterparts at the catalytic site rather than the substrate binding site (Nelson, 1998), consistent with the effectiveness of this particular class of inhibitors.

Ketoconazole had similar but not identical effects on the worms as cholesterol depletion. Defects were observed in growth, reproduction, and gonadal migration. Interestingly, cholesterol deprivation synergized with ketoconazole, increasing the potency of lower concentrations of ketoconazole on worm phenotypes (Table 2). These results suggest that ketoconazole targets signaling pathways that require cholesterol. As ketoconazole and the other imidazole drugs are known to target p450 enzymes in

other organisms, it seems likely that these drugs may alter the balance of cholesterol metabolites in the worm by regulating cholesterol processing by p450 enzymes. However, the interaction may also be indirect. In this regard, it would be illuminating to monitor by mass spectrometry the sterol profile of worms grown in the presence and absence of ketoconazole.

Notably, we did not observe inappropriate dauer formation or molting defects in worms grown on plates containing ketoconazole in any conditions tested. Inappropriate dauer formation and molting defects have been reported previously in approximately 5% of worms grown in the absence of cholesterol (Yochem *et al.*, 1999; Gerisch *et al.*, 2001). It is possible that different cholesterol metabolites are required for dauer, gonadal migration, and molting, and that ketoconazole treatment alters the concentration of a metabolites required for gonadal migration, but not those required for molting and dauer formation. Alternatively, dauer and molting defects might be observed in worms grown on ketoconazole plates under conditions different than those tested. Indeed, we noticed considerable phenotypic variability depending on type of media used (Table 5). Furthermore, ketoconazole may have a tissue-specific distribution in worms, such that it is not present in cells in which cholesterol metabolism is required for inappropriate dauer and molting phenotypes.

It has been suggested from genetic analyses that gonadal migration is controlled by an endocrine signaling pathway (Gerisch *et al.*, 2001). Interestingly, 24% of worms grown on 50 μM ketoconazole displayed altered gonandal migration, and this phenotype could be suppressed by a worm strain containing a null-allele of *daf-12 (rh61rh411)* (Table 7). These data suggest that ketoconazole might block an enzyme required for the synthesis of a *daf-12* ligand. The differences in sterol profiles of worms grown in the presence and absence of ketocoanzole may yield insight into the chemical nature of the putative *daf-12* ligand. In addition, it will be interesting to compare the sterol profile

of worms grown on ketoconazole with that of *daf-9* mutants (a p450 enzyme), as these worms also have defects in gonadal migration (Gerisch *et al.*, 2001; Jia *et al.*, 2002).

The *daf-12* null allele did not suppress the growth and reproductive phenotypes observed on plates containing ketoconazole but lacking cholesterol (Table 8). It is likely that cholesterol is required for additional unidentified signaling pathways. The isolation, mapping, and cloning of the genes suppressing the ketoconazole, cholesterol depletion, and *let-767* growth and reproductive phenotypes will be interesting in this regard. Although such a suppression screen would yield drug transporter or other proteins involved in degrading or removing ketoconazole from the worms, it should be possible to identify complementation groups that specifically alter *C. elegans* cholesterol signaling by testing strains for their ability to suppress the *let-767* and cholesterol depletion phenotypes. Clearly, ketoconazole and related drugs appear to be excellent tools to probe *C. elegans* sterol metabolism genetically.

Alternatively, the mechanism of ketoconazole action can be addressed by functional genomics. Preliminary experiments (Figure 7) with microarray analysis identified groups of genes that were upregulated in the presence of ketoconazole. Interestingly, of the 57 genes whose expression was upregulated in worms grown on plates containing ketoconazole by microarrays, six were cytochrome p450 enzymes. The increased expression of these six p450 enzymes was confirmed by qRT PCR (Figure 8); in other experiments, we identified 24 additional p450 enzymes whose expression was upregulated by ketoconazole. We found that the expression of multiple sub-families of p450 enzymes was affected by ketoconazole, but that the effects were selective within each sub-family. This is surprising given the greater than 60% identity shared by family members and their close genomic localization. For example, only five of the twelve 13A p450 family members displayed increased expression in the presence of the drug, even though the genes coding for nine sub-family members can be found within a single

cosmid (Figure 8B). It will be interesting to determine the purpose of this markedly increased p450 expression.

A whole genome RNAi based screen could be performed to identify gene products that make worms more or less sensitive to ketoconazole. Potentially interesting genes include nuclear receptors and the cytochrome p450 enzymes. It is thought that mammalian nuclear receptors respond to drugs and induce cytochrome p450 enzymes that metabolize these chemical compounds (Xie and Evans, 2001). Perhaps the induction of p450 expression observed in worms grown on ketoconazole is regulated by nuclear receptors. In addition, if p450 enzymes play a critical role in metabolizing ketoconazole, knocking out p450 expression should make the worms more sensitive to low concentrations of ketoconazole.

The experiments discussed in this chapter demonstrate that pharmacological tools can be used to gain insight into *C. elegans* biology and further support the hypothesis that cholesterol metabolites may serve as the basis for a class of nuclear receptor ligands in *C. elegans.* Clearly, the identification of such cholesterol-derived ligands is required to confirm this hypothesis. These ligands may be for the nuclear receptor *daf-12*and for other nuclear receptors and signaling proteins, as ketoconazole seemed to affect both *daf-12* dependent and independent signaling pathways (Table 8). The mapping of genetic suppressors of ketoconazole, cholesterol depletion, and *let-767*, the worm homologue of 17β-HSD, should not only help identify endogenous *C. elegans* ligands, but also help understand ligand biosynthesis and nuclear receptor regulation within a physiological context.

Thus, the identification of ligands in combination with the excellent genetic and developmental tools of *C. elegans*, should yield considerable insights into the physiological regulation of nuclear receptor function: the regulation of ligand production and the manner in which nuclear receptors are expressed in specific tissues, interact with

tissue specific cofactors, and bind to particular DNA sequences. Insights gained from studying these questions in *C. elegans* should be applicable to the study of more complex metazoans.

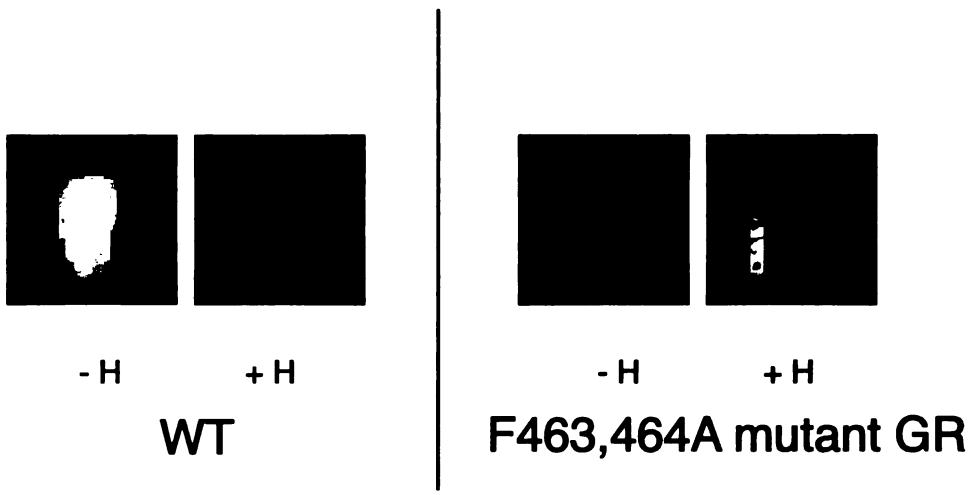
Appendix 1: Localization of GR in the absence of hormone with a mutated NES.

Introduction:

Several models have been suggested to explain the localization of GR in the absence of hormone. One possibility is that GR is actively exported from the nucleus in the absence of hormone. Hormone binding inhibits this nuclear export, allowing GR to accumulate in the nucleus. One nuclear export sequence (NES) has been identified in the DNA binding domain of GR. This sequence has been shown to be required for GR shuttling in the presence of hormone and GR redistribution to the cytoplasm during hormone withdrawal. The NES consists of residues 445-480, highly conserved residues that have been shown to play a crucial role in DNA binding.

Results and Discussion:

To examine a role of these residues in the localization of GR in the absence of hormone, we used site directed mutagenesis to convert the two conserved phenylalanine residues to alanine residues (F463A and F464A). These mutations have been shown to abolish the nuclear export activity of GR during hormone withdrawal (Black *et al.*, 2001; Holaska *et al.*, 2001). However, these mutations had no effect on the localization of GR observed in the absence of hormone (Appendix 1, Figure 1). It remains possible that another as yet unidentified GR NES could maintain GR in the cytoplasm in the absence of hormone.



Appendix 1, Figure 1: Mutations in the GR NES have no affect on GR localization in the absence of hormone. CV1 cells were transfected with plasmids pEGFP-N795 or pEGFP-N795 F463, 464A. Twenty-four hr after transfection, cells were treated with 10μM Dex (+H) or EtOH control (-H) for 4 hr before localization was assessed by microscopy.

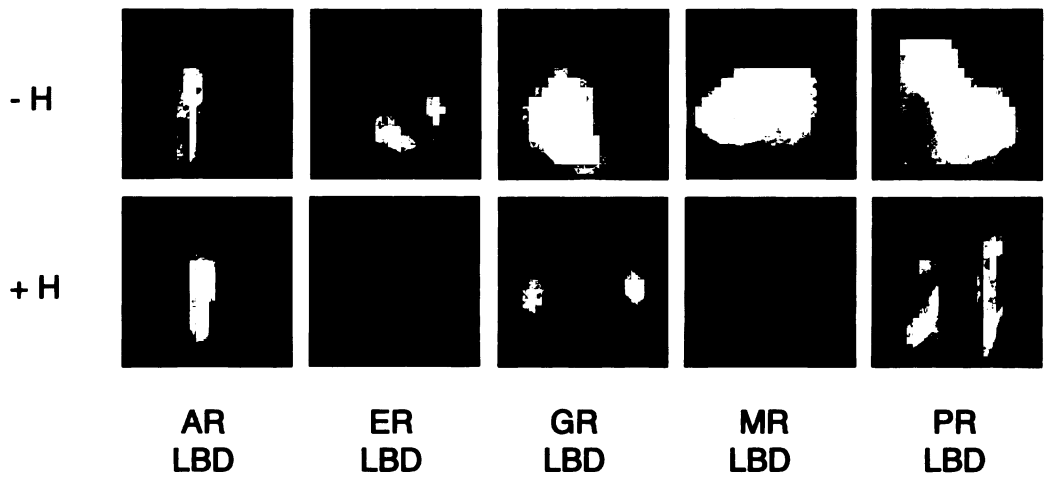
Appendix 2: NL2 activity in other members of the steroid hormone receptor family.

Introduction:

Different members of the steroid receptor family display different equilibrium intracellular distributions. GR, AR, PRB and MR are primarily cytoplasmic in the absence of hormone and nuclear in the presence of hormone. ER and PRA are nuclear and cytoplasmic in the absence of hormone and nuclear in the presence of hormone (Picard and Yamamoto, 1987; Simental *et al.*, 1991; Fejes-Toth *et al.*, 1998; Htun *et al.*, 1999; Lim *et al.*, 1999). NL1 is conserved between all of these proteins. The conservation of NL2, however, is not known.

Results and Discussion:

To determine if AR, ER, MR, and PR have a NL2, we fused the LBD of each protein to two copies of GFP and observed the localization of these fusion proteins in CV1 cells. As shown in Figure 1 of Appendix 2, GR, AR, PR and MR had a NL2, while ER did not. In light of the conserved NL2 activity in these receptors, it will be interesting to determine if importin 7 and importin 8 can bind to the NL2 of these other steroid hormone receptors.



Appendix 2: AR, GR, MR, PR but not ER have a NL2. CV1 cells were transfected with plasmids pEGFP^2-AR LBD, pEGFP^2-ER LBD, pEGFP^2-GR LBD, pEGFP^2-MR LBD, or pEGFP^2-PR LBD. Twenty four hr after transfection, cells transfected with each steroid receptor ligand binding domain were treated with the appropriate hormone at 10 μM (+H) or EtOH (-H). GR was treated with Dexamethasone, ER with estradiol, PR with progesterone, MR with dexamethasone, and AR with dihydroxytestosterone.

References:

Adam, S.A., Marr, R.S., and Gerace, L. (1990). Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. J Cell Biol *111*, 807-816.

Aitchison, J.D., Blobel, G., and Rout, M.P. (1996). Kap104p: a karyopherin involved in the nuclear transport of messenger RNA binding proteins. Science *274*, 624-627.

Aspock, G., Kagoshima, H., Niklaus, G., and Burglin, T.R. (1999). Caenorhabditis elegans has scores of hedgehog-related genes: sequence and expression analysis. Genome Res *9*, 909-923.

Baumann, C.T., Lim, C.S., and Hager, G.L. (1999). Intracellular localization and trafficking of steroid receptors. Cell Biochem Biophys *31*, 119-127.

Black, B.E., Holaska, J.M., Rastinejad, F., and Paschal, B.M. (2001). DNA binding domains in diverse nuclear receptors function as nuclear export signals. Curr Biol *11*, 1749-1758.

Bohen, S.P., and Yamamoto, K.R. (1993). Isolation of Hsp90 mutants by screening for decreased steroid receptor function. Proc Natl Acad Sci U S A *90*, 11424-11428.

Bohnsack, M.T., Czaplinski, K., and Gorlich, D. (2004). Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. Rna *10*, 185-191.

Bohnsack, M.T., Regener, K., Schwappach, B., Saffrich, R., Paraskeva, E., Hartmann, E., and Gorlich, D. (2002). Exp5 exports eEF1A via tRNA from nuclei and synergizes with other transport pathways to confine translation to the cytoplasm. Embo J *21*, 6205-6215.

Brelivet, Y., Kammerer, S., Rochel, N., Poch, O., and Moras, D. (2004). Signature of the oligomeric behaviour of nuclear receptors at the sequence and structural level. EMBO Rep *5*, 423-429.

Brown, G., Li, J., Lee, S.A., and Thomas, J.H. (2003). Mutations in the nuclear hormone receptor DAF-12 and its putative coregulator DIN-1 suppress the phenotypes of ncr mutants. International Worm Meeting, UCLA, Los Angeles, California, Abstract 266.

Brownawell, A.M., and Macara, I.G. (2002). Exportin-5, a novel karyopherin, mediates nuclear export of double-stranded RNA binding proteins. J Cell Biol *156*, 53-64.

Calado, A., Treichel, N., Muller, E.C., Otto, A., and Kutay, U. (2002). Exportin-5-mediated nuclear export of eukaryotic elongation factor 1A and tRNA. Embo J *21*, 6216-6224.

Cassada, R.C., and Russell, R.L. (1975). The dauerlarva, a post-embryonic developmental variant of the nematode Caenorhabditis elegans. Dev Biol *46*, 326-342.

Crowder, C.M., Westover, E.J., Kumar, A.S., Ostlund, R.E., Jr., and Covey, D.F. (2001). Enantiospecificity of cholesterol function in vivo. J Biol Chem *276*, 44369-44372.

Dahlman-Wright, K., Siltala-Roos, H., Carlstedt-Duke, J., and Gustafsson, J.A. (1990). Protein-protein interactions facilitate DNA binding by the glucocorticoid receptor DNA-binding domain. J Biol Chem *265*, 14030-14035.

Darimont, B.D., Wagner, R.L., Apriletti, J.W., Stallcup, M.R., Kushner, P.J., Baxter, J.D., Fletterick, R.J., and Yamamoto, K.R. (1998). Structure and specificity of nuclear receptor-coactivator interactions. Genes Dev *12*, 3343-3356.

Davis, L.I. (1995). The nuclear pore complex. Annu Rev Biochem *64*, 865-896.

Dean, K.A., von Ahsen, O., Gorlich, D., and Fried, H.M. (2001). Signal recognition particle protein 19 is imported into the nucleus by importin 8 (RanBP8) and transportin. J Cell Sci *114*, 3479-3485.

Dittmar, K.D., and Pratt, W.B. (1997). Folding of the glucocorticoid receptor by the reconstituted Hsp90-based chaperone machinery. The initial hsp90.p60.hsp70-dependent step is sufficient for creating the steroid binding conformation. J Biol Chem *272*, 13047-13054.

Dodd, M.H.I., and Dodd, J.M. (1976). The biology of metamorphosis. Academic Press: New York.

Engelhardt, D. (1994). Steroid biosynthesis inhibitors in Cushing s syndrome. Clin Investig *72*, 481-488.

Evans, R.M. (1988). The steroid and thyroid hormone receptor superfamily. Science *240*, 889-895.

Fejes-Toth, G., Pearce, D., and Naray-Fejes-Toth, A. (1998). Subcellular localization of mineralocorticoid receptors in living cells: effects of receptor agonists and antagonists. Proc Natl Acad Sci U S A *95*, 2973-2978.

Feng, W., and Hopper, A.K. (2002). A Los1p-independent pathway for nuclear export of intronless tRNAs in Saccharomycescerevisiae. Proc Natl Acad Sci U S A *99*, 5412-5417.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature *391*, 806-811.

Freedman, L.P., Yoshinaga, S.K., Vanderbilt, J.N., and Yamamoto, K.R. (1989). In vitro transcription enhancement by purified derivatives of the glucocorticoid receptor. Science *245*, 298-301.

Freeman, B.C., Felts, S.J., Toft, D.O., and Yamamoto, K.R. (2000). The p23 molecular chaperones act at a late step in intracellular receptor action to differentially affect ligand efficacies. Genes Dev *14*, 422-434.

Freeman, B.C., and Yamamoto, K.R. (2001). Continuous recycling: a mechanism for modulatory signal transduction. Trends Biochem Sci *26*, 285-290.

Galigniana, M.D., Radanyi, C., Renoir, J.M., Housley, P.R., and Pratt, W.B. (2001). Evidence that the peptidylprolyl isomerase domain of the hsp90-binding immunophilin FKBP52 is involved in both dynein interaction and glucocorticoid receptor movement to the nucleus. J Biol Chem *276*, 14884-14889.

Gametchu, B., and Harrison, R.W. (1984). Characterization of a monoclonal antibody to the rat liver glucocorticoid receptor. Endocrinology *114*, 274-279.

Gaunt, R. (1975). History of the Adrenal Cortex. In: Adrenal Gland, vol. VI, eds. R.O. Greep and E.B. Astwood, Washington, DC: American Physiological Society, 1-12.

Gerisch, B., Weitzel, C., Kober-Eisermann, C., Rottiers, V., and Antebi, A. (2001). A hormonal signaling pathway influencing C. elegans metabolism, reproductive development, and life span. Dev Cell *1*, 841-851.

Gietz, R.D., and Woods, R.A. (2002). Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol *350*, 87-96.

Gissendanner, C.R., Crossgrove, K., Kraus, K.A., Maina, C.V., and Sluder, A.E. (2004). Expression and function of conserved nuclear receptor genes in Caenorhabditis elegans. Dev Biol *266*, 399-416.

Golden, J.W., and Riddle, D.L. (1984). The Caenorhabditis elegans dauer larva: developmental effects of pheromone, food, and temperature. Dev Biol *102*, 368-378.

Gorlich, D., Dabrowski, M., Bischoff, F.R., Kutay, U., Bork, P., Hartmann, E., Prehn, S., and Izaurralde, E. (1997). A novel class of RanGTP binding proteins. J Cell Biol *138*, 65-80.

Gorlich, D., and Kutay, U. (1999). Transport between the cell nucleus and the cytoplasm. Annu Rev Cell Dev Biol *15*, 607-660.

Gorlich, D., Pante, N., Kutay, U., Aebi, U., and Bischoff, F.R. (1996). Identification of different roles for RanGDP and RanGTP in nuclear protein import. Embo J *15*, 5584-5594.

Gotoh, O. (1998). Divergent structures of Caenorhabditis elegans cytochrome P450 genes suggest the frequent loss and gain of introns during the evolution of nematodes. Mol Biol Evol *15*, 1447-1459.

Gwizdek, C., Ossareh-Nazari, B., Brownawell, A.M., Doglio, A., Bertrand, E., Macara, I.G., and Dargemont, C. (2003). Exportin-5 mediates nuclear export of minihelix-containing RNAs. J Biol Chem *278*, 5505-5508.

Gwizdek, C., Ossareh-Nazari, B., Brownawell, A.M., Evers, S., Macara, I.G., and Dargemont, C. (2004). Minihelix-containing RNAs mediate exportin-5-dependent nuclear export of the double-stranded RNA-binding protein ILF3. J Biol Chem *279*, 884-891.

Hache, R.J., Tse, R., Reich, T., Savory, J.G., and Lefebvre, Y.A. (1999). Nucleocytoplasmic trafficking of steroid-free glucocorticoid receptor. J Biol Chem *274*, 1432-1439.

He, F., and Jacobson, A. (1995). Identification of a novel component of the nonsense-mediated mRNA decay pathway by use of an interacting protein screen. Genes Dev *9*, 437-454.

Heinlein, C.A., and Chang, C. (2004). Androgen receptor in prostate cancer. Endocr Rev *25*, 276-308.

Hieb, W.F., and Rothstein, M. (1968). Sterol requirement for reproduction of a free-living nematode. Science *160*, 778-780.

Holaska, J.M., Black, B.E., Love, D.C., Hanover, J.A., Leszyk, J., and Paschal, B.M. (2001). Calreticulin Is a receptor for nuclear export. J Cell Biol *152*, 127-140.

Hopper, A.K., Schultz, L.D., and Shapiro, R.A. (1980). Processing of intervening sequences: a new yeast mutant which fails to excise intervening sequences from precursor tRNAs. Cell *19*, 741-751.

Htun, H., Holth, L.T., Walker, D., Davie, J.R., and Hager, G.L. (1999). Direct visualization of the human estrogen receptor alpha reveals a role for ligand in the nuclear distribution of the receptor. Mol Biol Cell *10*, 471-486.

Huh, W.K., Falvo, J.V., Gerke, L.C., Carroll, A.S., Howson, R.W., Weissman, J.S., and O Shea, E.K. (2003). Global analysis of protein localization in budding yeast. Nature *425*, 686-691.

Imamoto, N., Shimamoto, T., Takao, T., Tachibana, T., Kose, S., Matsubae, M., Sekimoto, T., Shimonishi, Y., and Yoneda, Y. (1995). In vivo evidence for involvement of a 58 kDa component of nuclear pore-targeting complex in nuclear protein import. Embo J *14*, 3617-3626.

Iniguez-Lluhi, J.A., Lou, D.Y., and Yamamoto, K.R. (1997). Three amino acid substitutions selectively disrupt the activation but not the repression function of the glucocorticoid receptor N terminus. J Biol Chem *272*, 4149-4156.

Iovine, M.K., Watkins, J.L., and Wente, S.R. (1995). The GLFG repetitive region of the nucleoporin Nup116p interacts with Kap95p, an essential yeast nuclear import factor. J Cell Biol *131*, 1699-1713.

Jakel, S., Albig, W., Kutay, U., Bischoff, F.R., Schwamborn, K., Doenecke, D., and Gorlich, D. (1999). The importin beta/importin 7 heterodimer is a functional nuclear import receptor for histone H1. Embo J *18*, 2411-2423.

Jakel, S., and Gorlich, D. (1998). Importin beta, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells. Embo J *17*, 4491-4502.

Jakel, S., Mingot, J.M., Schwarzmaier, P., Hartmann, E., and Gorlich, D. (2002). Importins fulfil a dual function as nuclear import receptors and cytoplasmic chaperones for exposed basic domains. Embo J *21*, 377-386.

Jeong, J., and McMahon, A.P. (2002). Cholesterol modification of Hedgehog family proteins. J Clin Invest *110*, 591-596.

Jia, K., Albert, P.S., and Riddle, D.L. (2002). DAF-9, a cytochrome P450 regulating C. elegans larval development and adult longevity. Development *129*, 221-231.

Johnston, S.R., and Dowsett, M. (2003). Aromatase inhibitors for breast cancer: lessons from the laboratory. Nat Rev Cancer *3*, 821-831.

Kadowaki, T., Chen, S., Hitomi, M., Jacobs, E., Kumagai, C., Liang, S., Schneiter, R., Singleton, D., Wisniewska, J., and Tartakoff, A.M. (1994). Isolation and characterization of Saccharomyces cerevisiae mRNA transport-defective (mtr) mutants. J Cell Biol *126*, 649-659.

Kaffman, A., and O Shea, E.K. (1999). Regulation of nuclear localization: a key to a door. Annu Rev Cell Dev Biol *15*, 291-339.

Kaffman, A., Rank, N.M., O Neill, E.M., Huang, L.S., and O Shea, E.K. (1998a). The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus. Nature *396*, 482-486.

Kaffman, A., Rank, N.M., and O Shea, E.K. (1998b). Phosphorylation regulates association of the transcription factor Pho4 with its import receptor Pse1/Kap121. Genes Dev *12*, 2673-2683.

Kalderon, D., Roberts, B.L., Richardson, W.D., and Smith, A.E. (1984). A short amino acid sequence able to specify nuclear location. Cell *39*, 499-509.

Kamath, R.S., and Ahringer, J. (2003). Genome-wide RNAi screening in Caenorhabditis elegans. Methods *30*, 313-321.

Kamath, R.S., Fraser, A.G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., Welchman, D.P., Zipperlen, P., and Ahringer, J. (2003). Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. Nature *421*, 231-237.

Kino, T., Souvatzoglou, E., De Martino, M.U., Tsopanomihalu, M., Wan, Y., and Chrousos, G.P. (2003). Protein 14-3-3sigma interacts with and favors cytoplasmic subcellular localization of the glucocorticoid receptor, acting as a negative regulator of the glucocorticoid signaling pathway. J Biol Chem *278*, 25651-25656.

Kose, S., Imamoto, N., Tachibana, T., Shimamoto, T., and Yoneda, Y. (1997). Ran-unassisted nuclear migration of a 97-kD component of nuclear pore-targeting complex. J Cell Biol *139*, 841-849.

Kuersten, S., Ohno, M., and Mattaj, I.W. (2001). Nucleocytoplasmic transport: Ran, beta and beyond. Trends Cell Biol *11*, 497-503.

Kuervers, L.M., Jones, C.L., O Neil, N.J., and Baillie, D.L. (2003). The sterol modifying enzyme LET-767 is essential for growth, reproduction and development in Caenorhabditis elegans. Mol Genet Genomics *270*, 121-131.

Kurisaki, A., Kose, S., Yoneda, Y., Heldin, C.H., and Moustakas, A. (2001). Transforming growth factor-beta induces nuclear import of Smad3 in an importin-beta1 and Ran-dependent manner. Mol Biol Cell *12*, 1079-1091.

Kurzchalia, T.V., and Ward, S. (2003). Why do worms need cholesterol? Nat Cell Biol *5*, 684-688.

Kutay, U., Izaurralde, E., Bischoff, F.R., Mattaj, I.W., and Gorlich, D. (1997). Dominant-negative mutants of importin-beta block multiple pathways of import and export through the nuclear pore complex. Embo J *16*, 1153-1163.

Kuwabara, P.E., Lee, M.H., Schedl, T., and Jefferis, G.S. (2000). A C. elegans patched gene, ptc-1, functions in germ-line cytokinesis. Genes Dev *14*, 1933-1944.

Lefstin, J.A., and Yamamoto, K.R. (1998). Allosteric effects of DNA on transcriptional regulators. Nature *392*, 885-888.

Li, S., Armstrong, C.M., Bertin, N., Ge, H., Milstein, S., Boxem, M., Vidalain, P.O., Han, J.D., Chesneau, A., Hao, T., Goldberg, D.S., Li, N., Martinez, M., Rual, J.F., Lamesch, P., Xu, L., Tewari, M., Wong, S.L., Zhang, L.V., Berriz, G.F., Jacotot, L., Vaglio, P., Reboul, J., Hirozane-Kishikawa, T., Li, Q., Gabel, H.W., Elewa, A., Baumgartner, B., Rose, D.J., Yu, H., Bosak, S., Sequerra, R., Fraser, A., Mango, S.E., Saxton, W.M., Strome, S., Van Den Heuvel, S., Piano, F., Vandenhaute, J., Sardet, C., Gerstein, M., Doucette-Stamm, L., Gunsalus, K.C., Harper, J.W., Cusick, M.E., Roth, F.P., Hill, D.E., and Vidal, M. (2004). A map of the interactome network of the metazoan C. elegans. Science *303*, 540-543.

Lim, C.S., Baumann, C.T., Htun, H., Xian, W., Irie, M., Smith, C.L., and Hager, G.L. (1999). Differential localization and activity of the A- and B-forms of the human progesterone receptor using green fluorescent protein chimeras. Mol Endocrinol *13*, 366-375.

Liu, J., and DeFranco, D.B. (2000). Protracted nuclear export of glucocorticoid receptor limits its turnover and does not require the exportin 1/CRM1-directed nuclear export pathway. Mol Endocrinol *14*, 40-51.

Lu, T.T., Repa, J.J., and Mangelsdorf, D.J. (2001). Orphan nuclear receptors as eLiXiRs and FiXeRs of sterol metabolism. J Biol Chem *276*, 37735-37738.

Lund, E., Guttinger, S., Calado, A., Dahlberg, J.E., and Kutay, U. (2004). Nuclear export of microRNA precursors. Science *303*, 95-98.

Makishima, M., Lu, T.T., Xie, W., Whitfield, G.K., Domoto, H., Evans, R.M., Haussler, M.R., and Mangelsdorf, D.J. (2002). Vitamin D receptor as an intestinal bile acid sensor. Science *296*, 1313-1316.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and et al. (1995). The nuclear receptor superfamily: the second decade. Cell *83*, 835-839.

Matyash, V., Geier, C., Henske, A., Mukherjee, S., Hirsh, D., Thiele, C., Grant, B., Maxfield, F.R., and Kurzchalia, T.V. (2001). Distribution and transport of cholesterol in Caenorhabditis elegans. Mol Biol Cell *12*, 1725-1736.

Melchior, F., and Gerace, L. (1998). Two-way trafficking with Ran. Trends Cell Biol *8*, 175-179.

Merris, M., Wadsworth, W.G., Khamrai, U., Bittman, R., Chitwood, D.J., and Lenard, J. (2003). Sterol effects and sites of sterol accumulation in Caenorhabditis elegans: developmental requirement for 4alpha-methyl sterols. J Lipid Res *44*, 172-181.

Michalik, L., Desvergne, B., and Wahli, W. (2003). Peroxisome proliferator-activated receptors beta/delta: emerging roles for a previously neglected third family member. Curr Opin Lipidol *14*, 129-135.

Moore, M.S., and Schwoebel, E.D. (2000). Nuclear Import in Digitonin-Permeabilized Cells. In: Current protocols in cell biology, ed. J.S. Bonifacino, New York: John Wiley, 11.17.11-11.17.17.

Morehouse, H., Buratowski, R.M., Silver, P.A., and Buratowski, S. (1999). The importin/ karyopherin Kap114 mediates the nuclear import of TATA-binding protein. Proc Natl Acad Sci U S A *96*, 12542-12547.

Murphy, C.T., McCarroll, S.A., Bargmann, C.I., Fraser, A., Kamath, R.S., Ahringer, J., Li, H., and Kenyon, C. (2003). Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. Nature *424*, 277-283.

Nachury, M.V., and Weis, K. (1999). The direction of transport through the nuclear pore can be inverted. Proc Natl Acad Sci U S A *96*, 9622-9627.

Nagoshi, E., Imamoto, N., Sato, R., and Yoneda, Y. (1999). Nuclear import of sterol regulatory element-binding protein-2, a basic helix-loop-helix-leucine zipper (bHLH-Zip)-containing transcription factor, occurs through the direct interaction of importin beta with HLH-Zip. Mol Biol Cell *10*, 2221-2233.

Nelson, D.R. (1998). Metazoan cytochrome P450 evolution. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol *121*, 15-22.

Nieman, L.K. (2002). Medical therapy of Cushing s disease. Pituitary *5*, 77-82.

Nigg, E.A. (1997). Nucleocytoplasmic transport: signals, mechanisms and regulation. Nature *386*, 779-787.

Paddison, P.J., Silva, J.M., Conklin, D.S., Schlabach, M., Li, M., Aruleba, S., Balija, V., O Shaughnessy, A., Gnoj, L., Scobie, K., Chang, K., Westbrook, T., Cleary, M., Sachidanandam, R., McCombie, W.R., Elledge, S.J., and Hannon, G.J. (2004). A resource for large-scale RNA-interference-based screens in mammals. Nature *428*, 427-431.

Pante, N., and Aebi, U. (1995). Exploring nuclear pore complex structure and function in molecular detail. J Cell Sci Suppl *19*, 1-11.

Pemberton, L.F., Rosenblum, J.S., and Blobel, G. (1999). Nuclear import of the TATA-binding protein: mediation by the karyopherin Kap114p and a possible mechanism for intranuclear targeting. J Cell Biol *145*, 1407-1417.

Perlmann, T., and Wrange, O. (1988). Specific glucocorticoid receptor binding to DNA reconstituted in a nucleosome. Embo J *7*, 3073-3079.

Picard, D. (1993). Steroid-binding domains for regulating the functions of heterologous proteins in cis. Trends Cell Biol *3*, 278-280.

Picard, D., Salser, S.J., and Yamamoto, K.R. (1988). A movable and regulable inactivation function within the steroid binding domain of the glucocorticoid receptor. Cell *54*, 1073-1080.

Picard, D., and Yamamoto, K.R. (1987). Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. Embo J *6*, 3333-3340.

Pratt, W.B., Silverstein, A.M., and Galigniana, M.D. (1999). A model for the cytoplasmic trafficking of signalling proteins involving the hsp90-binding immunophilins and p50cdc37. Cell Signal *11*, 839-851.

Reboul, J., Vaglio, P., Rual, J.F., Lamesch, P., Martinez, M., Armstrong, C.M., Li, S., Jacotot, L., Bertin, N., Janky, R., Moore, T., Hudson, J.R., Jr., Hartley, J.L., Brasch, M.A., Vandenhaute, J., Boulton, S., Endress, G.A., Jenna, S., Chevet, E., Papasotiropoulos, V., Tolias, P.P., Ptacek, J., Snyder, M., Huang, R., Chance, M.R., Lee, H., Doucette-Stamm, L., Hill, D.E., and Vidal, M. (2003). C. elegans ORFeome version 1.1: experimental verification of the genome annotation and resource for proteome-scale protein expression. Nat Genet *34*, 35-41.

Reinke, V., Smith, H.E., Nance, J., Wang, J., Van Doren, C., Begley, R., Jones, S.J., Davis, E.B., Scherer, S., Ward, S., and Kim, S.K. (2000). A global profile of germline gene expression in C. elegans. Mol Cell *6*, 605-616.

Rexach, M., and Blobel, G. (1995). Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. Cell *83*, 683-692.

Riddle, D.L., and Albert, P.S. (1997). Genetic and environmental regulation of dauer larva development. In: *C. elegans* II, ed. D.L. Riddle, Blumenthanal., T., Meyer, B., and Priess,D., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 739-768.

Riddle, D.L., Blumenthal, T., Meyer, B.J., and Priess, J.R. (1997). Introduction to *C. elegans*. In: *C. elegans II*, eds. D.L. Riddle, T. Blumenthal, B.J. Meyer, and J.R. Priess, Cold Spring Harbor, NY: Cold Spring Harbor Press, 1-22.

Rogatsky, I., Zarember, K.A., and Yamamoto, K.R. (2001). Factor recruitment and TIF2/ GRIP1 corepressor activity at a collagenase-3 response element that mediates regulation by phorbol esters and hormones. Embo J *20*, 6071-6083.

Rosenblum, J.S., Pemberton, L.F., and Blobel, G. (1997). A nuclear import pathway for a protein involved in tRNA maturation. J Cell Biol *139*, 1655-1661.

Saporita, A.J., Zhang, Q., Navai, N., Dincer, Z., Hahn, J., Cai, X., and Wang, Z. (2003). Identification and characterization of a ligand-regulated nuclear export signal in androgen receptor. J Biol Chem *278*, 41998-42005.

Savory, J.G., Hsu, B., Laquian, I.R., Giffin, W., Reich, T., Hache, R.J., and Lefebvre, Y.A. (1999). Discrimination between NL1- and NL2-mediated nuclear localization of the glucocorticoid receptor. Mol Cell Biol *19*, 1025-1037.

Sawicki, M.W., Erman, M., Puranen, T., Vihko, P., and Ghosh, D. (1999). Structure of the ternary complex of human 17beta-hydroxysteroid dehydrogenase type 1 with 3-hydroxyestra-1,3,5,7-tetraen-17-one (equilin) and NADP+. Proc Natl Acad Sci U S A *96*, 840-845.

Schena, M., and Yamamoto, K.R. (1988). Mammalian glucocorticoid receptor derivatives enhance transcription in yeast. Science *241*, 965-967.

Seedorf, M., and Silver, P.A. (1997). Importin/karyopherin protein family members required for mRNA export from the nucleus. Proc Natl Acad Sci U S A *94*, 8590-8595.

Sharp, R.B., and Penning, T.M. (1988). Inhibition of progesterone synthesis in normal and transformed human placental cells by tight binding inhibitors of 3 beta-hydroxysteroid dehydrogenase. Steroids *51*, 441-457.

Shim, Y.H., Chun, J.H., Lee, E.Y., and Paik, Y.K. (2002). Role of cholesterol in germ-line development of Caenorhabditis elegans. Mol Reprod Dev *61*, 358-366.

Simental, J.A., Sar, M., Lane, M.V., French, F.S., and Wilson, E.M. (1991). Transcriptional activation and nuclear targeting signals of the human androgen receptor. J Biol Chem *266*, 510-518.

Sonino, N. (1987). The use of ketoconazole as an inhibitor of steroid production. N Engl J Med *317*, 812-818.

Spaete, R.R., and Mocarski, E.S. (1985). Regulation of cytomegalovirus gene expression: alpha and beta promoters are trans activated by viral functions in permissive human fibroblasts. J Virol *56*, 135-143.

Stade, K., Ford, C.S., Guthrie, C., and Weis, K. (1997). Exportin 1 (Crm1p) is an essential nuclear export factor. Cell *90*, 1041-1050.

Stoffler, D., Fahrenkrog, B., and Aebi, U. (1999). The nuclear pore complex: from molecular architecture to functional dynamics. Curr Opin Cell Biol *11*, 391-401.

Strom, A.C., and Weis, K. (2001). Importin-beta-like nuclear transport receptors. Genome Biol *2*, REVIEWS3008.

Suzuki, S., Kurata, N., Nishimura, Y., Yasuhara, H., and Satoh, T. (2000). Effects of imidazole antimycotics on the liver microsomal cytochrome P450 isoforms in rats: comparison of in vitro and ex vivo studies. Eur J Drug Metab Pharmacokinet *25*, 121-126.

Sym, M., Basson, M., and Johnson, C. (2000). A model for niemann-pick type C disease in the nematode Caenorhabditis elegans. Curr Biol *10*, 527-530.

Takizawa, C.G., Weis, K., and Morgan, D.O. (1999). Ran-independent nuclear import of cyclin B1-Cdc2 by importin beta. Proc Natl Acad Sci U S A *96*, 7938-7943.

Tang, Y., Ramakrishnan, C., Thomas, J., and DeFranco, D.B. (1997). A role for HDJ-2/ HSDJ in correcting subnuclear trafficking, transactivation, and transrepression defects of a glucocorticoid receptor zinc finger mutant. Mol Biol Cell *8*, 795-809.

Tatar, M., Bartke, A., and Antebi, A. (2003). The endocrine regulation of aging by insulin-like signals. Science *299*, 1346-1351.

Thomas, B.J., and Rothstein, R. (1989). Elevated recombination rates in transcriptionally active DNA. Cell *56*, 619-630.

Thompson, E.B., Simons, S.S., Jr., and Harmon, J.M. (1981). Deacylcortivazol, a potent glucocorticoid with unusual structure and unusual anti-leukemic cell activity. Adv Exp Med Biol *138*, 315-324.

Titov, A.A., and Blobel, G. (1999). The karyopherin Kap122p/Pdr6p imports both subunits of the transcription factor IIA into the nucleus. J Cell Biol *147*, 235-246.

Tomkins, G.M. (1975). The metabolic code. Science *189*, 760-763.

Tomkins, G.M., and Martin, D.W., Jr. (1970). Hormones and gene expression. Annu Rev Genet *4*, 91-106.

Turpin, P., Ossareh-Nazari, B., and Dargemont, C. (1999). Nuclear transport and transcriptional regulation. FEBS Lett *452*, 82-86.

Urbina, J.A., Vivas, J., Visbal, G., and Contreras, L.M. (1995). Modification of the sterol composition of Trypanosoma (Schizotrypanum) cruzi epimastigotes by delta 24(25)-sterol methyl transferase inhibitors and their combinations with ketoconazole. Mol Biochem Parasitol *73*, 199-210.

Van Gilst, M., Gissendanner, C.R., and Sluder, A.E. (2002). Diversity and function of orphan nuclear receptors in nematodes. Crit Rev Eukaryot Gene Expr *12*, 65-88.

Weis, K. (1998). Importins and exportins: how to get in and out of the nucleus [published erratum appears in Trends Biochem Sci 1998 Jul;23(7):235]. Trends Biochem Sci *23*, 185-189.

Wicks, S.R., Yeh, R.T., Gish, W.R., Waterston, R.H., and Plasterk, R.H. (2001). Rapid gene mapping in Caenorhabditis elegans using a high density polymorphism map. Nat Genet *28*, 160-164.

Xiao, Z., McGrew, J.T., Schroeder, A.J., and Fitzgerald-Hayes, M. (1993). CSE1 and CSE2, two new genes required for accurate mitotic chromosome segregation in Saccharomyces cerevisiae. Mol Cell Biol *13*, 4691-4702.

Xie, W., and Evans, R.M. (2001). Orphan nuclear receptors: the exotics of xenobiotics. J Biol Chem *276*, 37739-37742.

Yamamoto, K.R. (1985). Steroid receptor regulated transcription of specific genes and gene networks. Annu Rev Genet *19*, 209-252.

Yamamoto, K.R., Darimont, B.D., Wagner, R.L., and Iniguez-Lluhi, J.A. (1998). Building transcriptional regulatory complexes: signals and surfaces. Cold Spring Harb Symp Quant Biol *63*, 587-598.

Yamamoto, K.R., Stampfer, M.R., and Tomkins, G.M. (1974). Receptors from glucocorticoid-sensitive lymphoma cells and two clases of insensitive clones: physical and DNA-binding properties. Proc Natl Acad Sci U S A *71*, 3901-3905.

Yano, R., Oakes, M., Yamaghishi, M., Dodd, J.A., and Nomura, M. (1992). Cloning and characterization of SRP1, a suppressor of temperature-sensitive RNA polymerase I mutations, in Saccharomyces cerevisiae. Mol Cell Biol *12*, 5640-5651.

Yi, R., Qin, Y., Macara, I.G., and Cullen, B.R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev *17*, 3011-3016.

Yochem, J., Tuck, S., Greenwald, I., and Han, M. (1999). A gp330/megalin-related protein is required in the major epidermis of Caenorhabditis elegans for completion of molting. Development *126*, 597-606.

Zhang, W., Ramamoorthy, Y., Kilicarslan, T., Nolte, H., Tyndale, R.F., and Sellers, E.M. (2002). Inhibition of cytochromes P450 by antifungal imidazole derivatives. Drug Metab Dispos *30*, 314-318.