Role of CDC37 in the Structural Proofreading of ERBB Receptor Tyrosine Kinases

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ROLE OF CDC37 IN THE STRUCTURAL PROOFREADING OF ERBB RECEPTOR
TYROSINE KINASES

By
Wenjun Liu

A DISSERTATION

Submitted to the Faculty
of the University of Miami
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the degree of Doctor of Philosophy

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A dissertation submitted in partial fulfillment of
the requirements for the degree of
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PROOFREADING OF ERBB RECEPTOR TYROSINE
KINASES

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Folding and maturation of most protein kinases require chaperone assistance. In higher eukaryotes, CDC37 is the predominant cochaperone that facilitates the transfer of kinase clients to Hsp90. Kinase recognition is thought to occur through the N-terminal domain, which has, thus far, eluded structure determination. Client processing requires phosphorylation of the N-terminal tail at Ser13 by the protein kinase, CK2 (casein kinase 2). How phosphorylation alters the molecular properties of CDC37 is not understood. We show that phosphorylation at Ser13 induces a large shift towards a more compact structure, based on ANS fluorescence, while modestly increasing the secondary structure. Moreover, this transition requires interactions of the N-terminal domain and the remainder of CDC37. The stabilizing property of the phosphorylation event can be recreated in trans by a (phospho-Ser13) peptide derived from the N-terminal tail. However, the phosphorylation-induced transition is not dependent on the transferred phosphate group but rather on the loss of serine-like properties at position 13. The complete absence of the N-terminal tail results in a reduced secondary structure and unresponsiveness to subsequent addition of peptides. The N-terminal tail may therefore serve as an intramolecular chaperone that ensures that CDC37 assumes one of two
readily interconvertible states in a manner that impacts the interaction between the client binding N-domain and the MC-domains, involved in dimerization and Hsp90 binding.

To study the role of CDC37 in structural proofreading with its client engaged, we adopted ERBB3 as a model kinase client. ERBB3 is one of four members of the ERBB receptor tyrosine kinase family. Distinct from the other ERBB members, ERBB3 has a highly impaired kinase domain with only 0.1% kinase activity compared to fully activated EGFR. Lys 723 is part of the kinase domain, and its mutation, K723M, largely diminishes the ATP binding ability of ERBB3. Despite its potential structural perturbation, K723M is successfully expressed in both insect and mammalian cells, representing itself as a valuable model for folded kinase with defects. In stable cell lines expressing either the WT or K723M ERBB3, K723M has similar mRNA levels and protein stability as WT, but demonstrates a significantly reduced steady state protein levels. In transiently transfected lines, the cell surface fraction of K723M is stable, whereas its total protein undergoes extensive degradation. We also presented that the K723M mutant of ERBB3 forms a tighter association with the CDC37/Hsp90 chaperone complex, compared to that of the wild type ERBB3. Studies of CFTR (Cystic fibrosis transmembrane conductance regulator) mutant ΔF508 suggest a correlation between tighter chaperone association and higher ER associated degradation. Indeed we observed low overall production of K723M. The phosphoserine mutants of CDC37 are deficient in recognizing the structural perturbation at the kinase domain of K723M. This work elucidates CDC37 as not only a kinase sorting module, but a structural defect discriminator in the structural proofreading.
As a vital molecule in structural proofreading, CDC37 processes many kinases that are oncogenic or critical for cancer development. Cancer treatment via CDC37 targeting is being proposed in many circumstances. However, no available direct inhibitor has been disclosed yet. We demonstrated that Withaferin A (WA) interacts with CDC37 and disrupts the Hsp90/CDC37 complex with ERBB2. The exact binding affinity is not clear, but the effects on protein downregulation, signaling attenuation and proliferation are characterized. The ERBB3 receptor contributes to resistance in treatments that target ERBB2 receptor tyrosine kinase, and its levels represent an overall risk factor for unfavorable disease outcomes in breast cancer. In the absence of classic catalytic activity, it is not a target of pan ERBB kinase inhibitors. WA has established broad anti-cancer properties through several modes of action and has been shown to be effective against triple negative breast cancers. We found that ERBB2 overexpression renders cells hypersensitive to WA. While ERBB2 downregulation is one aspect of WA treatment, it is not causal for the elevated sensitivity. In addition WA also targets ERBB3, a crucial and highly synergistic activity in ERBB2 overexpression. WA diminishes ERBB3 receptor levels and its constitutive activation as well as its associated activation of AKT activation. The simultaneous targeting of ERBB2 and ERBB3 renders cells vulnerable, even in BT474, where direct ERBB2 targeting with Lapatinib is less effective. In conclusion, WA or its derivatives may therefore serve as a low toxicity addition to supplement ERBB2 targeted therapeutics, especially in cases where ERBB3 involvement is driving resistance or reduced overall sensitivity.

The post folding structural conformation of a kinase is essential for CDC37 recognition. There is no easy method to differentiate folding status and activation states of kinases.
We collaborated with Dr. James Wilson to develop fluorescence activation state specific (FAST) probes against ERBB family receptor tyrosine kinases. We contributed to the conceptual design based on ATP cocrystal structures of ERBB kinase domains and the evaluation of these novel molecules in cell settings. With the incorporation of an ERBB targeting pharmacophore and optical report element, the probe emission increases due to the hydrophobic environment and restricted geometry of ERBB kinase domain upon binding. This facilitates the analysis of receptor states at low occupancy without the removal of unbound probes. With these “Turn-On probes”, conformational properties of ERBB receptor tyrosine kinases can be readily assessed after sorting by CDC37.
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Chapter 1 Introduction

1.1 CDC37 & Structural proofreading

1.1.1 Concept of structural proofreading for kinases

Many cellular proteins are known to not spontaneously fold but instead require the assistance of other proteins called “molecular chaperones”. For transmembrane receptor kinases, these processes of folding and maturation are carried out by two distinguishable pools of chaperone/cochaperones, residing in the ER lumen and cytosol respectively. Upon synthesis, nascent kinase domains first undergo folding, which is mainly carried out by Hsp40/70 and their accessory cochaperone complex\(^7\) that mostly work on the exposed hydrophobic area of the substrate.\(^8\) Since the machinery of Hsp40/70 does not convey specificity towards its substrates (referred to as clients in the chaperone field), this folding assistance is functionally generic. Upon completion of the generic folding assistance, clients are handed over to the Hsp90 dimers in an “adapter” dependent manner for later folding and quality control (see review\(^9\)). Quality control as a more inclusive term includes checkpoints against glycosylation, disulfide bond formation, structural conformation, or other side chain modification. In the current study, I will refer the quality control of structural confirmation as the structural proofreading (see Figure 1.1 for a conceptual illustration). For kinases, the cochaperone cell division cycle protein 37 (CDC37) functions as an adapter molecule by recognizing its kinase clients after completion of the generic folding, and subsequently recruiting the Hsp90 dimer. Therefore, Hsp90 binds with a large but specific subset of clients.\(^10\) The coordination of successive cochaperone entry/exit, transition between various complexes of changing
composition and signaling events such as phosphorylation/dephosphorylation constitutes late folding assistance as well as structural proofreading (see review\(^{11}\)). Cycling of Hsp90 in its ATP-bound (clamped down) versus ADP-bound (relaxed or unclamped) state results in a binding/release equilibrium\(^{12}\) that represents the efforts of later folding as well as the decision step in structural proofreading for hard to fold cytoplasmic domains of clients. For membrane receptors, this coincides with the completion of ER assisted folding (ERAF) and export to the Golgi, as opposed to ER assisted degradation (ERAD) and extraction from the ER. Generally, the folding landscape on the ER can sometimes be traversed quickly. Small proteins such as the Semliki Forest virus capsid protein can fold in approximately 50 ms.\(^{13}\) However, for larger and more complex proteins that require
more time for structural proofreading, for example, the human coagulation factors V and VIII, folding can take several minutes to hours to complete.\textsuperscript{14}

Great efforts have been made to identify the participating molecules, explore the progression of proofreading complex, and reveal the signaling events in several major chaperone/cochaperones which together lead to a better understanding about the structural proofreading of kinases. However, molecular details about the recognition of kinase clients are still largely unknown. How can the structural proofreading machinery recognize a large quantity of kinases that vary in functions and structures? How is the structural error in the client kinase identified by the structural proofreading machinery? These are the two major questions that need to be addressed in the field of structural proofreading.

1.1.2 CDC37 biology

1.1.2.1 Molecular basis of CDC37 in kinase processing

CDC37 was first identified in Saccharomyces cerevisiae as a cell division cycle regulating protein.\textsuperscript{15} CDC37 is a kinase specific cochaperones that serves as an adaptor or scaffold molecule facilitating interactions of kinases and Hsp90.\textsuperscript{16} The classic view of CDC37 domain functions include its middle domain (M-domain), which is responsible for Hsp90 recruitment and CDC37 dimerization\textsuperscript{17-19}, and its N-terminal domain (N-domain), which interacts with kinase clients\textsuperscript{20, 21} (see Figure 1.2A). The library of CDC37 clients covers over 350 proteins that are almost exclusively ATP binding folds (mainly, but not exclusively, kinases), including many oncogenic kinases, such as Raf, AKT, and ERBB2.\textsuperscript{22} CDC37 also interacts with a limited number of non-kinase clients, including
androgen receptor, reverse transcriptase, and the guanine nucleotide exchange factor Vav3. So far only two X-ray structures of CDC37 are available, based on its M-domain and part of its C-terminal domain (C-domain), leaving the whole N-domain unresolved. The N-domain of CDC37 is hypothesized to be structurally uncommitted and flexible in order to accommodate its wide spectrum of clients.

As an example of the structural proofreading complex, Figure 1.2B shows the EM structure of the in vivo assembled CDK4/Hsp90/CDC37 complex isolated from Sf9 cells. This electron microscopy (EM) reconstruction provides the first experimental view of a client loaded Hsp90/CDC37 complex. Due to the limitation of resolution of negative-stain EM, the exact domain orientations and the details of

Figure 1.2. (A) A classic view of the domain functions of CDC37 (Taken from Miyata, 2009). The structurally unresolved N-domain interacts with kinases. The M-domain is responsible for Hsp90 recruitment and homodimerization. (B) EM structure of the in vivo assembled CDK4/Hsp90/CDC37 complex with a 1:2:1 composition. (Taken from Vaughan and Pearl, 2006)
interactions between each molecule are not well resolved, however a model has been generated. The complex is comprised of CDK4(1)/Hsp90(2)/CDC37(1), and the general architecture of this triple complex is presented. Note that CDK4 in this complex represents the client kinase domain, i.e. the ERBB3 kinase domain in our case.

Advances in the understanding of CDC37 functions have revealed limitations of the classic working model. For a subset of clients, CDC37 was indicated to process its clients independently of Hsp90. At the same time, CDC37 is not able to compensate for loss of Hsp90 function, indicating distinct and non-redundant roles of CDC37 and Hsp90. The defect in v-Src folding in yeast with deleted Sti1 (an Hsp90 cochaperone in eukaryote) could be rescued not only by overexpression of full length CDC37, but also to a considerable extent by overexpression of the N-domain of CDC37. On the other hand, the C-domain of CDC37 is required for the association of Vav3 with CDC37. Taken together, these findings suggest that the classic isolated domain functions might not be completely accurate.

1.1.2.2 Phosphorylation of CDC37

The function of CDC37 is proposed to be regulated by phosphorylation at its Ser 13 residue by CK2. Mutations of the Ser 13 site in yeast lead to severe growth deficiency and depletion of CDC37 clients. Phosphorylation of this site has been reported to be critical for client recognition, as phosphorylation incapable mutation S13A reduces the interaction of CDC37 with multiple kinase clients. Notably, the phosphomimetic mutations S13D and S13E also reduce normal client binding, indicating that it is the structural feature surrounding the serine site rather than the phosphor group that
determines the ability of CDC37 to bind clients. However it is still not clear how phosphorylation regulates the action of CDC37 on the molecular level. The ability of CDC37 to establish very defined interactions with its clients, yet retain a high degree of structural flexibility represents a feature also found in intrinsically disordered proteins (IDP) or protein domains. Furthermore, computational assessments indicate a strong correlation between intrinsic disorder and occurrence of post-translational modifications, including phosphorylation, acetylation, and O-glycosylation. Posttranslational modifications can induce transitions between order and disorder and thus regulate protein or domain interactions. In chapter 2, we will closely examine interference of Ser13 phosphorylation with the structural property of CDC37. Other than serine phosphorylation, CDC37 is also phosphorylated on Tyr4 and Tyr298 by YES. Phosphorylation of both Y4 and Y298 by YES abolishes CDC37-kinase association with a certain class of kinases, indicating a role of the C-domain of CDC37 in kinase recognition/binding.

1.1.3 Recognition by CDC37 as the first layer quality control

Since the CDC37-client interaction involves the select recognition of a specific client class, this interaction may be regarded not only as folding or maturation assistance but as an early stage in quality control. Only qualified kinase folds are selectively recognized by CDC37, and redirected for further folding and structural proofreading. The ability for this sorting remains in the recognition of structural elements that identify a protein as a kinase. Despite the large quantity, CDC37 clients are all ATP binding pocket containing proteins, suggesting that the ATP binding pocket is the structural element. Variations in the ATP binding pocket across different classes of kinases and non-kinases exist. Thus it is
necessary that CDC37 is capable of adopting a variety of ATP pockets and differentiating a kinase with structural perturbations from the wild type conformation. In Chapter 3, we will explore the intricacies of molecular recognition for a defective kinase, and the potential consequences.

1.2 ERBB receptor tyrosine kinase

1.2.1 ERBB and ERBB relevant diseases

The ERBB family of receptor tyrosine kinase (RTKs) is a group of structurally related type I RTKs that consists of four members: EGFR/ERBB1, ERBB2/Neu/HER2, ERBB3/HER3, and ERBB4/HER4. All ERBB receptors have a conserved structural composition including an extracellular domain (ECD), a transmembrane span, juxtamembrane region, a kinase domain (including N and C lobes), and a carboxyl terminal tail. The extracellular domain is ready for ligand binding with the exception of ERBB2 whose tethered ECD is deficient in binding ligands. Excess ERBB signaling due to elevated receptor levels, mutation, or autocrine stimulation has been implicated in a wide variety of solid tumors. High EGFR expression was found in the majority of carcinomas and ERBB2 gene amplification accounts for over 30% of metastatic breast lesions. While ERBB2 is a strong contributor to breast cancer development through its downstream MAPK pathway, its oncogenic potency is highly dependent on the available complement of hetero dimerizing ERBB family members. Particularly, the kinase impaired ERBB3 receptor complements the MAPK centered signal transduction of ERBB2 by its exceptionally signal into the PI3K/AKT pathway (see Figure 1.3).
To date, targeted therapies for ERBB driven cancers exist in the form of monoclonal antibodies (Herceptin (trastuzumab), Pertuzumab, both targeting ERBB2) or small molecule kinase inhibitors towards ERBB tyrosine kinase (e.g. Lapatinib, targeting ERBB2, EGFR, and ERBB4). Two of the most successful anti-ERBB drugs, Herceptin and Lapatinib, show high initial response rates, but long term clinical studies also indicate a high rate of relapse and resistance through diverse mechanisms including elevated ERBB3 activity. While never overexpressed in isolation, ERBB3 has clearly emerged as a key player in resistance against ERBB2 or EGFR directed treatments. Several monoclonal antibodies (MM121, AV-203) against ERBB3 are currently under evaluation in clinical trials.

Despite extensive studies of ERBB in cancer scenarios, fundamental knowledge of ERBB in the normal setting is very limited. ERBB targeted therapies (such as Herceptin) are associated with a high risk of the development of cardiotoxic cardiomyopathy in...
patients. This observation in clinic led to important studies that elucidated the importance of the neuregulin (NRG)/ERBB signaling pathways in heart physiology and cardiovascular function. Developmental studies of mice lacking NRG-1\textsuperscript{9}, ERBB2\textsuperscript{10,12} or ERBB4\textsuperscript{49,50} indicated the important role of NRG-ERBB signaling in early heart development, as embryos died during embryogenesis and displayed heart malformations. A recent study in rats and mice at different stages of pregnancy demonstrated that protein expressions of different NRG1 isoforms, and the levels of phosphorylated ERBB2 and ERBB4 significantly increased in the LV (left ventricular) tissue after 1-2 weeks of pregnancy.\textsuperscript{51} Treatments with Lapatinib partially inhibited ERBB signaling, and led to a premature maternal death in about 25% of the total tested animals, as well as induced abnormalities in LV physiology, probably due to affected mitochondria functions.\textsuperscript{52}

1.2.2 ERBB3 is an ideal model for studying structural proofreading

A large portion of critical mammalian kinases require interactions with CDC37 and Hsp90.\textsuperscript{53} This interaction can reside in the nascent state during the process of maturation alone or in both the mature and nascent state of the protein, possibly in qualitatively different association states. The multiple binding states of CDC37 to kinases make studies on structural proofreading difficult, since the interaction between clients and Hsp90 complex does not exclusively comes from the nascent pool. Regarding ERBB family receptor kinases, the nascent forms of EGFR and ERBB2 associate at the ER with a complex comprising the Hsp90 chaperone and the kinase-dedicated adaptor. Upon glycosylation and delivery to the plasma membrane, the mature form of ERBB2 remains associated with the chaperone. Recent studies in our lab showed that unlike ERBB2, the interactions of CDC37, as well as Hsp90, with wild type ERBB3 reside exclusively in the
nascent state.\textsuperscript{54, 55} Therefore, the study of chaperone interactions with ERBB3 provides an experimental route to the selective study of interactions in the nascent state and therefore to the study of structural proofreading of ERBB receptors.

Fundamental biochemical studies recently revealed that ERBB3 binds ATP with a Kd of \( \sim 1.1 \) µM and clustered ERBB3 on lipid vesicle is able to carry out autophosphorylation in trans.\textsuperscript{54} Preactivated and purified ERBB3 is capable of phosphorylating peptide substrates.\textsuperscript{55} The X-ray crystal structure of ERBB3 incorporates adenylylimidodiphosphate (AMP-PNP), but presents an inactive conformation (Figure 1.4). The ATP binding pocket of the ERBB3 kinase domain is supposed to be constitutively saturated with ATP under normal physiological conditions, due to a low Kd at approximately 1.1 µM. Lys 723 is part of the ATP binding pocket\textsuperscript{56} of ERBB3 kinase domain and is conserved in all tyrosine kinases. The mutation K723M at the kinase domain completely abolishes ATP binding and kinase activity. Despite the potential structural perturbation, the K723M mutant folds and is expressed in insect and mammalian cells.\textsuperscript{57, 58} Both K723M and its equivalent K721M, for EGFR, were reported to express on cell surface and respond to ligand stimulation.\textsuperscript{58, 59} Based on Figure 1.4. The K723M mutation of ERBB3 kinase. Shown here is the structure of AMP-PNP bound ERBB3 kinase domain based on PDB: 3LMG. The helix C (blue) is open positioned and the activation loop (purple) blocks the access of ATP representing an inactive conformation. The across tyrosine kinase conserved Lys 723 is part of the ATP binding pocket and labeled red.
these facts, the well characterized K723M mutation of ERBB3 serves as a structural perturbation to query the nature of CDC37 recognition. We will challenge the recognition of ERBB3 by CDC37 with the K723M mutation in chapter 3.

1.3 Targeting CDC37 for cancer treatments

1.3.1 Targeting chaperone/cochaperones for cancer and other diseases

Hsp90 regulates many cellular processes that are implicated in cancer, such as proliferation, cell cycle progression, survival, invasion, angiogenesis, and metastasis. In cancer settings, many Hsp90 clients such as ERBBs, PINK, FGFRs, AKT/PKB, Raf, and Bcr-Abl are oncoproteins that are either mutated or over-expressed. It has also been demonstrated that many oncogenic signals are addict to Hsp90 activities. A lot of effort has been employed to develop Hsp90 inhibitors for targeted therapy, among which Geldanamycin and its derivative 17-AAG are the most successful. However, the wide spectrum of Hsp90 clients is as much a risk as an asset for Hsp90 targeting inhibitors, since administration of these drugs would impair those Hsp90 clients not involved in carcinogenesis but critical for cell survival.

Furthermore, studies have expanded attention from the core chaperone to peripheral co-chaperones. The ΔF508, single amino acid deletion in the cystic fibrosis transmembrane conductance regulator (CFTR), is found in almost 70% of CF patients and leads to complete

Figure 1.5. Multiprotein complex based proofreading and the bind/release equilibrium for CFTR. (Taken from Wang, 2006)
ERAD based degradation of this vital, gated chloride channel. Yet the affected subunit is known by crystallography to show only subtle deviations in structure as a consequence of the deletion. For CFTR (ΔF508), the specificity of the Hsp90 interaction is achieved through layers of more client or client-class specific co-chaperones, including a range of novel and class specific co-chaperones that are part of the proofreading complex.62,63 The class specific Aha1 kinase associates with and modifies the proofreading machinery to favor ATP hydrolysis by Hsp90, client release, and degradation (Figure 1.5). As a proof of concept study of targeting chaperone/cochaperones, Aha1 knockdown phenotypically rescued CFTR (ΔF508) by effectively redefining the threshold for successful proofreading.2 Note that while certainly more selective than Hsp90, Aha1 is not strictly CFTR specific.

1.3.2 CDC37 is a novel entry for targeted therapies

Although overexpression of CDC37 occurs in limited cancers and at relatively modest levels, it has been demonstrated as a promising entry point for targeted therapy. Genome-wide functional screening identifies CDC37 as a crucial factor for KIT oncogenic expression in gastrointestinal stromal tumors.64 With no validated inhibitors against CDC37, only knock down of CDC37 has been tested in cell models. Silencing CDC37 by siRNA or shRNA impairs the stability or production of many oncogenic kinases, such as ERBB2, cRAF, CDK4, CDK6, and AKT in various cancer lines.65-67 The anticipated advantage of targeting CDC37 as opposed to Hsp90 is that the client selectivity of the cochaperone CDC37 is predominantly directed to protein kinases. Therefore, the targeting of CDC37 is expected to have most potential in kinase-addicted cancers. We searched the literature for any possible CDC37 inhibitors. The Hsp90-CDC37 complex
was reported to be a potential molecular target of Withaferin A. \(^{68}\) Additionally, a computational model suggests potential docking site on CDC37 for WA. \(^{69}\) In chapter 5 we will examine this possibility and evaluate the anti-cancer effects of WA in breast cancer cell models.
Chapter 2 Phosphorylated and unphosphorylated serine 13 of CDC37 stabilize distinct interactions between its client and Hsp90 binding domains

2.1 Summary

Folding and maturation of most protein kinases require chaperone assistance. In higher eukaryotes, CDC37 is the predominant cochaperone that facilitates the transfer of kinase clients to Hsp90. Kinase recognition is thought to occur through the N-terminal domain, which has, thus far, eluded structure determination. Client processing also requires the phosphorylation of the N-terminal tail at Ser13 by protein kinase CK2 (casein kinase 2). How phosphorylation alters the molecular properties of CDC37 is not understood. We show that the phosphorylation at Ser13 induces a large shift toward a more compact structure, based on ANS fluorescence, while modestly increasing secondary structure. Moreover, this transition requires interactions of the N-terminal domain and the remainder of CDC37. The stabilizing property of the phosphorylation event can be recreated in trans by a (phospho-Ser13) peptide derived from the N-terminal tail. However, the phosphorylation-induced transition is not dependent on the transferred phosphate group but rather the loss of serine-like properties at position 13. The complete absence of the N-terminal tail results in reduced secondary structure and unresponsiveness to subsequent addition of peptides. The N-terminal tail may therefore serve as an intramolecular chaperone that ensures that CDC37 assumes one of two readily interconvertible states in a manner that impacts the interaction of the client binding N-domain and the MC-domains, involved in dimerization and Hsp90 binding.
2.2 Background remarks

For many cellular proteins, initial folding and the subsequent maturation require complex cellular machinery. This machinery, consisting of an array of chaperones and cochaperones, engages its substrates, also referred to as clients, in a sequential manner. Chaperones that act in early client folding (e.g., Hsp70) are distinct from those that assist at later stages of maturation. These later stages may involve broad specificity chaperones, such as Hsp90, as well as protein class specific cochaperones, and transitions occur by client handover in large chaperone complexes (reviewed in ref^{70}). In the case of the majority of kinases or select ATP binding domains, the critical cochaperone is CDC37 (cell division cycle protein 37). Since CDC37-client interaction involves the select recognition of the client class, this interaction may be regarded not only as folding or maturation assistance but also as an early stage in quality control. Failure of a client to successfully pass through the successive rearrangements of the various client–chaperone complexes may result in its degradation. As demonstrated for mutations in CFTR (cystic fibrosis transmembrane conductance regulator), severe functional deficiencies may arise due to relatively minor alterations in client structure and highly stringent quality control.²

Functionally, CDC37 is thought to act as an adaptor or scaffold molecule facilitating the interaction of clients with Hsp90.¹ Since its first identification in 1980, the library of CDC37 clients has quickly expanded to cover over 350 proteins that involve almost exclusively ATP binding folds (mainly, but not exclusively, kinases).²² At least for a subset of kinase clients, CDC37 is capable of facilitating client processing independent of Hsp90.²⁷,²⁸ Its kinase binding domain is sufficient to carry out this task, but this
interaction has been proposed to reflect a less supervised mode of client maturation that is physiologically superseded by Hsp90-dependent processing due to the low concentrations of CDC37 and abundance of Hsp90. CDC37 does interact with select targets outside the kinase family, such as androgen receptor, reverse transcriptase, or the guanine nucleotide exchange factor Vav3. However, at least for the latter, the functional region of CDC37 is distinct from its kinase binding domain. Many oncogenic kinases heavily rely on CDC37 activity. Silencing of CDC37 expression by siRNA or shRNA depletes clients such as ERBB2, cRAF, CDK4, CDK6, and AKT in human colon, breast, and prostate cancer lines. Overexpression of CDC37 occurs in various cancers but at relatively modest levels, possibly reflecting its transitory involvement with a small intermittent pool for most of its clients. Targeting the interactions between CDC37 and its kinase clients is, therefore, a potential entry point for therapeutics aimed at individual overactive kinases or kinase pathways. However, the molecular events underlying molecular recognition of such a broad range of clients as well as the assembly and disassembly of Hsp90 complexes remain poorly understood.

In-solution studies identified an inherently weak ability of CDC37 to dimerize with a micromolar dissociation constant. Current models of CDC37 action (reviewed in ref) involve the recognition of its client by a CDC37 dimer, binding of dimeric Hsp90, and the subsequent dissociation of one CDC37 molecule to generate a relatively stable 1:1:2 complex of client, CDC37, and the Hsp90 dimer. Of the three major domains of CDC37 (Figure 2.1), the N-terminal domain engages kinase clients and the central M-domain is responsible for the recruitment of Hsp90 and homodimerization of CDC37. CDC37 function is regulated through phosphorylation at Ser13 by protein
kinase CK2 (casein kinase 2). In studies of yeast CDC37, mutations of the CK2 target site result in severe growth deficiency and depletion of CDC37 clients.\textsuperscript{31, 32}

This suggests that CDC37 function as a class specific cochaperone is largely not redundant. Available structural data on the M-domain and part of the C-terminal segment\textsuperscript{17, 26} provide insight into the nature of the CDC37–Hsp90 complex, but the N-domain is not resolved in this structure. Figure 2.1 shows a simplified version of the model that has been derived from several pieces of crystallographic data.\textsuperscript{17} It features a symmetric complex containing two molecules of CDC37. This model is complemented by an EM reconstruction of a 1:1:2 complex.\textsuperscript{3} However, the latter also failed to assign the location and mode of interaction of the N-domain relative to client. Biochemical data suggest that the dephosphorylation of the N-terminus of CDC37 is important for the maturation complex to proceed. This dephosphorylation within the Hsp90 multiprotein complex is highly context-dependent and requires PP5 phosphatase.\textsuperscript{30} However, the mechanism by which

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**Figure 2.1.** Domain organization of CDC37. (A) Only the middle domain (M) and part of the C-terminal domain (C) are structurally resolved by X-ray crystallography and depicted as solid blocks. The N-domain is responsible for kinase client recognition but is not structurally resolved. The N-terminal peptide contains an acidic substrate sequence for CK2 and the phosphorylation site at serine 13. (B) Model of CDC37/CDC37* dimer bound to HSP90 dimer and client. The representation is a simplified version of a model proposed by Vaughan et al.\textsuperscript{3} Modeled components are shown as spheres structured around the crystallographically confirmed backbone of the M and partial C-domain. Each M-domain interacts with an N-terminal ATP binding domain of HSP90.
phosphorylation achieves this objective is not understood, and it is not clear how CDC37 is capable of recognizing or identifying such a broad array of clients while rejecting some closely related kinases as clients.

The ability of CDC37 to establish defined interactions with its clients, yet retain a high degree of structural flexibility, represents a feature also found in intrinsically disordered proteins (IDP) or protein domains. For the most part, IDPs maintain mainly random coil or molten globule structure and manifest extraordinary structural flexibility and plasticity by sampling a variety of conformations that are in equilibrium. Alternatively, the ensembles represented in IDPs may also involve various alternative structured states that are in rapid equilibrium unless a specific state is selected out through modifications or interactions. Computational methods predict that over 30% of proteins in higher eukaryotes are IDPs or harbor long, intrinsically disordered segments defined as more than 50 consecutive residues. IDPs have been identified in key cellular processes such as transcription, signaling transduction, cell cycle control, and programmed cell death. There are already multiple cases in which either chaperone molecules function through their ID segment or IDPs are found to possess chaperone activity.

Computational assessments indicate a strong correlation between intrinsic disorder and the occurrence of posttranslational modifications, including phosphorylation, acetylation, and O-glycosylation. Post-translational modifications can induce transitions between order and disorder and thus regulate protein or domain interactions. In the before mentioned example of CFTR, channel gating is regulated by its normally structured R-domain, which competes against the heterodimerization of two nucleotide binding
domains, NBD1 and NBD2. The structural compactness of the R-domain is lost upon phosphorylation, thus favoring NBD heterodimerization and altering the state of the channel. The tasks being performed by CDC37, the proposed structural properties of the N-domain, and the mode of regulation by CK2 are consistent with those of a regulated IDP. In this study, we provide experimental evidence that phosphorylation causes significant changes in the level of compactness of CDC37, that this transition involves the tail region and the combined N and MC domains, and that the resulting state is characterized by a high degree of stability. The transition depends on the loss of serine-like side chain properties, not phosphorylation itself. The ability of the phosphorylated N-terminal tail to induce structural changes can be recapitulated qualitatively in trans, using an exogenous phosphopeptide derived from its N-terminus. However, the overall change in secondary structure is very small, indicating a rearrangement of pre-existing structural elements rather than a transition from a largely disordered to a globular state.

2.3 Materials and methods

Constructs and Proteins. The human ERBB2 and ERBB3 cDNA were expressed in a pFlag-myc-CMV-19 vector together with a C-terminal biotinylation acceptor peptide sequence (GLNDIFEAQKIEWHE). The Escherichia coli biotinyl transferase BirA was cloned into the pLXSN vector for cotransfection with biotinylation tagged constructs. The mammalian expression construct of HA-tagged CDC37 in the pKH3 vector was generously provided by Dr. Kerry Burnstein (University of Miami). From this construct, full length (1–378), Δ1–19 (20–378), N-terminal domain (1–148), and MC-domains (149–378) were derived and cloned into the bacterial expression vector pQE40. Point mutants including S13A, S13D, S13E, and S13C were created in pQE40 using the
Agilent Technologies QuikChange mutagenesis system. A CK2 construct for bacterial expression was directly purchased from Addgene (plasmid 27083: pDB1 (CK2alpha)). For all in vitro studies, both CDC37 and CK2 constructs were expressed in the DH12S strain of *E. coli* (Invitrogen) and purified by FPLC (Biorad) on a Ni-NTA column (CDC37) or glutathione affinity column (CK2), respectively. Proteins were finally dialyzed and stored in PBS. The isolated N-domain of CDC37 shows a high tendency to aggregate during dialysis and storage. To overcome this issue, the N-domain was eluted from the Ni-NTA column with PBS containing 2 mM EDTA and processed immediately for CD spectroscopy and ANS assays by spin column desalting (Princeton Separations, CS-900).

**In vitro phosphorylation/dephosphorylation.** In vitro phosphorylation of CDC37 by CK2 was carried out at 37 °C or room temperature for the indicated time periods. Reactions were close to complete after 3 h. The in vitro phosphorylation buffer contained 50 mM Tris-HCl, 10 mM MgCl2, 0.1 mM EDTA, 0.1 mM DTT, and 2.5 mM ATP. CDC37 concentrations were 1–6.5 μM with a 10:1 molar ratio of CDC37 to CK2. For studies on dephosphorylation efficiency, phosphorylation by CK2 was carried out for 3 h and blocked by the addition of 4, 5, 6, 7-tetrabromobenzotriazole (TBB). For samples that were denatured prior to dephosphorylation, the indicated detergents were added and samples were boiled for 5 min. All samples were diluted 50-fold into phosphate buffer containing a high concentration of calf intestine alkaline phosphatase (0.1 U/μL final concentration). Samples were incubated for 1 h at the indicated temperatures.

**Immunodepletion and immunoprecipitation.** To estimate the phosphorylation efficiency in vitro and in cell culture, three sequential immunodepletions of a
phosphorylation reaction or cell lysate, respectively, were carried out. CDC37-containing solutions were diluted into 500 μL of PBS and incubated with 4 μL of CDC37 pS13 antibody (Epitomics, 3600-1) and 15 μL of protein A/G agarose beads (Santa Cruz, sc-2003). After incubation on a rotator for 30 min and centrifugation, the supernatant was subjected to a second and third round of immunodepletion. Detection of the remaining soluble CDC37 was done by CDC37 antibody (Santa Cruz, sc-13129) on western blots.

For the study of cellular interactions of recombinant CDC37 with ERBB clients in cell lysate, 5 × 10⁵ BT474 cells were lysed in 500 μL of mild lysis buffer (20 mM Tris, 137 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA) containing 1 mM sodium orthovanadate and 1 mM PMSF and underwent predepletion with anti-His antibody. The cleared lysate was incubated with 15 pmol of in vitro phosphorylated recombinant CDC37, 5 μL of anti-His antibody (Genscript, A00186), and 20 μL of protein A/G beads. After 1 h of incubation, the beads were subjected to three washes with mild lysis buffer and analyzed by western blot using ERBB2 (Abcam, ab8054), ERBB3 (Santa Cruz, sc-285), or Hsp90 (Stressgen, SPA-840) antibodies.

For coimmunoprecipitation experiments that were selective for cell surface receptors, N-terminally Flag epitope-tagged ERBB2 and ERBB3 were transiently expressed in the MCF7 breast cancer cell line in 6-well plates. Anti-Flag antibody (Sigma, F1804) was added to the culture medium and incubated on ice for 30 min to prevent internalization. Free antibody was removed by three washes with cold PBS, and cells were lysed in mild lysis buffer. The lysate was incubated with protein A/G beads for 1 h on a slow speed rotor. Beads were washed three times with lysis buffer, and associated proteins were analyzed by SDS-PAGE and western blots.
For the coimmunoprecipitation using biotinylation tags, MCF7 cells were transfected with ERBB2-bio or ERBB3-bio, BirA, and, when indicated, HA-CDC37. The bio tag at the C-terminus of ERBBs encodes a biotinylation substrate peptide (GLNDIFEAQKIEWHE) for the BirA biotinyl transferase. The lysates were incubated with neutravidin agarose beads (Pierce, 29202) for 1 h. Were indicated, in vitro phosphorylated, *E. coli* expressed CDC37 was added to the incubation system. After the incubation, beads were washed three times with lysis buffer, and associated proteins were analyzed by western blots.

**CD spectrum.** CD spectra were collected either directly, using the phosphorylation reaction, or following removal of CK2. For CK2 removal, in vitro phosphorylation mixtures containing both CDC37 and CK2 or CK2 alone were incubated with glutathione agarose resin, and the supernatant was loaded onto gel filtration spin columns for rebuffering just prior to CD analysis. The reconstitution buffer for CD analysis contained only 10 mM potassium phosphate (pH 7.5) with or without 1 M urea. The spectra were collected on a Jasco CD spectrometer (model J-815). The spectra obtained this way matched those obtained by measurements after desalting alone followed by subtraction of the CK2 control spectrum. Due to the substantially lower loss during handling, the latter approach was adopted for routine measurements of point mutants. Peptides representing the 19 N-terminal residues of CDC37, including regular or phosphorylated serine in position 13, were synthesized by Biomatik. The peptide sequences were

MVDYSVWDHIEVSDDEDETC or MVDYSVWDHIEVpSDDEDETC, including an additional cysteine in position 20 for optional immobilization. For CD measurements, the peptides were reconstituted in 10 mM potassium phosphate buffer (pH 7.5) at a
concentration of 300 μg/mL. For the N-terminal domain, the protein was eluted from the Ni-NTA column in PBS with 2 mM EDTA and subjected to spin column buffer exchange into the respective buffers for CD spectroscopy or ANS assays.

**ANS assay.** The fluorescent spectrum of 1-anilinonaphthalene- 8-sulfonic acid (ANS) was determined in PBS in a Jasco fluorometer (model J-6500). As a reference, 10 μM ANS was incubated with 6 μM BSA, yielding maximal emission with an excitation wavelength of 380 nm. For CDC37 measurements, 20 μM ANS was incubated with 1 μM CDC37 in either phosphorylation buffer or PBS. For temperature control studies with DHFR, 0.64 μM DHFR and 4.62 μM CDC37 were incubated with and without a 10-fold molar excess of methotrexate. Synthetic peptides derived from the N-terminus of CDC37 were added at the ratio of 1:1 to the indicated CDC37 constructs.

**Secondary structure and disorder predictions.** The secondary structure prediction for CDC37 was obtained using the PSIPRED algorithm using default settings at the Protein Sequence Analysis Workbench server. The propensity of segments of CDC37 for structural disorder was predicted using the PONDR suite of algorithms using default settings. The analysis presented included four algorithms: VL3, VSL2, VLXT, and XL1-XT.

### 2.4 Results

#### 2.4.1 Assessment of *E. coli* expressed CDC37

For most of our studies, we relied on human CDC37, expressed as recombinant protein in *E. coli*. Bacterial expressed CDC37 has been used in prior studies. However, we wanted to ascertain that the recombinant protein we generated was a reasonable substitute.
for mammalian expressed CDC37 for our objectives. We therefore evaluated the ability of CDC37 to be phosphorylated by protein kinase CK2 (casein kinase 2) at Ser13 and the ability of recombinant CDC37 to recognize mammalian clients with a selectivity profile that matches that of endogenous mammalian CDC37. The ability of CK2 to recognize and selectively phosphorylate Ser13 of CDC37 in vitro was confirmed by western blot analysis using a CDC37/pSer13-specific antibody for detection (Figure 2.2A). In different studies using either 3- or 2-fold sequential immunodepletions, we found 78% (± 7) of endogenous CDC37 to be constitutively phosphorylated in mammalian cells (Figure 2.2B). For recombinant human CDC37, expressed in MCF7 cells, approximately 90% of CDC37 is phosphorylated or resides in complexes carrying at least one phosphorylated Ser13 (data not shown). Phosphorylation by CK2 is, therefore, reproducible in vitro and recreates a state that is representative of most of the CDC37 in a cellular setting.

As an additional functional test we evaluated the ability of recombinant CDC37 to bind Hsp90 and client. For Hsp90, clients fall into two categories: those requiring Hsp90 only during maturation and those associating with it also in the mature state. We previously showed that ERBB3 (HER3), a receptor tyrosine kinase of the EGFR family, falls into the first category, with only a very small portion of ERBB3 at any time being transiently associated with Hsp90 during maturation. By contrast, the closely related ERBB2 (HER2) receptor is a well-established representative of proteins requiring Hsp90 throughout their life cycle. Before we could utilize this knowledge as a functional test for recombinant CDC37, we needed to establish whether this Hsp90-derived interaction pattern also extended to CDC37. To this end, we expressed recombinant, biotinylation tagged ERBB2 or ERBB3 in MCF7 cells and measured the recovery of CDC37 or Hsp90
that bound to neutravidin resin purified receptors from cell lysate (Figure 2.2C).

Endogenous CDC37 was recovered at significantly higher yield bound to ERBB2 than that to ERBB3, suggesting that the previously established binding preference of Hsp90 carries over to CDC37. This preference is also evident for recombinant HA-tagged CDC37 expressed in MCF7 cells. In this case, the quantitative analysis is limited by the
strong differential impact of overexpressed CDC37 on the levels of cotransfected ERBB receptors. We validated this binding preference for the binding of *E. coli* expressed and in vitro phosphorylated CDC37 to biotin tagged receptors in cellular lysate. Therefore, *E. coli* derived CDC37 demonstrated a binding preference that matches that of endogenous CDC37. The same results were obtained when we compared recombinant CDC37 expressed in mammalian cells and its selective interaction with cell surface, and hence mature, receptors (data now shown) with the interaction of phosphorylated *E. coli* derived CDC37 and endogenous ERBB receptors in BT474 cells (Alimandi 1995, Kraus 1987) overexpressing both receptors in response to ERBB2 gene amplification (data not shown). The failure of ERBB3 and CDC37 to coimmunoprecipitate reflects a CDC37 dependence that is limited to transient interactions in the nascent state. This dependency is, however, apparent in ERBB3 levels when CDC37 is knocked down by siRNA (Figure 2.2D). We therefore concluded that *E. coli* generated CDC37 is a suitable substitute for mammalian expressed CDC37 in our studies based on its own phosphorylation and differential client binding properties.

**2.4.2 Phosphorylation at Ser 13 increases structural compactness of the N-terminus**

The high yield of in vitro phosphorylation by CK2 facilitates the analysis of its impact on the structural dynamics of CDC37. An analysis of CDC37 by circular dichroism spectroscopy indicates a high prevalence of α helices (Figure 2.3A). This finding fits secondary structure predictions and partial structural information available for CDC37. On the basis of a deconvolution of the spectrum using the CDpro package of algorithms and a reference data set of soluble proteins, α helices constitute approximately 80% of CDC37, with the remainder representing coiled coil segments.
CK2-mediated phosphorylation of CDC37 induced modest increases in helical content. For Figure 2.3A, samples had been depleted of CK2 by glutathione agarose prior to desalting and CD analysis. However, we observed the same shift when the contribution of CK2 was removed by subtraction of its CD spectrum (used for Figure 2.3B). While small in magnitude, the increases in helical content were highly reproducible between studies at 2.1 ± 0.3% (calculated from triplicate spectra of duplicate experimental sets). This increase occurred at the expense of random coil segments and translates into an estimated helix formation by approximately 10 additional amino acids. Interestingly, the increase is reversed when challenged with 1 M urea while the initially observed α-helical content remains intact (Figure 2.3B). This would suggest that phosphorylation induces structural changes that

Figure 2.3. Phosphorylation on Ser13 monitored by CD spectroscopy. (A) CDC37 is largely composed of α-helices (black). After overnight phosphorylation by CK2 at room temperature, α-helical content of CDC37 increases (red). Deletion of the first 19 amino acids causes a significant loss of α-helical content (blue). Spectra were obtained after CK2 removal on GSH columns and desalting. (B) The CD spectrum after overnight phosphorylation, desalting, and subtraction of CK2 spectrum gives matching results but higher recovery. Upon phosphorylation the α-helical content of CDC37 increases by 2 to 3%. (data representative of duplicate studies and triplicate spectral measurements). The gain in α-helical content but not the remaining secondary structure is destabilized when 1 M urea is added after overnight phosphorylation. For experiments involving urea, data below 205 nm are not suitable for analysis and were omitted.
involve a well defined region and is readily reversible without compromising the remainder of the protein structure. By contrast, CDC37 lacking the extreme N-terminal tail (CDC37 Δ1–19) shows a loss in helical content (Figure 2.3A) despite high stability and solubility of the purified protein.

To further explore the phosphorylation-induced transition in CDC37, we applied the fluorescent dye, 1-anilinonaphthalene-8-sulfonic acid (ANS). This amphiphilic dye shows a strong increase in fluorescence when it obtains access to hydrophobic pockets of a protein. It is, therefore, widely used to obtain a measure of protein compactness. However, the intensity of the ANS signal depends on at least two major parameters. The first is the access to interior binding sites for ANS, which is being utilized for measurements of compactness. However, in order to bind, ANS does need structural elements with which to interact, and an all-out loss in secondary structure can, therefore, also result in reduced signal. Prior studies on the molten globule state of several proteins, including recombinant human growth hormone, show maximum fluorescence in the molten globule state and minimal fluorescent in the fully folded native and unfolded states.

The fluorescence of full-length CDC37 (NMC, representing its individual domains) drops significantly following phosphorylation by CK2 (NMC, t = 14 h). Most of the decrease occurs within 3 h and is complete after 14 h at room temperature. The CDC37 Δ1–19 truncation mutant displays fluorescence similar to that of phosphorylated full-length CDC37, whereas the middle and C-terminal segments of CDC37 (MC) show very low ANS fluorescence. Together with the very favorable protein properties during synthesis and storage, this suggests a stable and compact structure for MC and would suggest that
the large variation in ANS signal is derived from phosphorylation-induced changes of the N-terminal domain (N). However, in isolation, the N-terminal segment does not display a high ANS signal. For direct comparison, Figure 2.4A also shows the calculated sum of both signals, which is comparable in concentration and composition to that for wild-type protein. The sum of the N and MC segments amounts to approximately 65% of the signal obtained from phosphorylated CDC37, or less than 30% of the unphosphorylated reference. Yet, in sharp contrast to the MC-domains, the N-domain

Figure 2.4. On the basis of ANS fluorescence, CDC37 becomes more compact upon phosphorylation. Legend is arranged in order of spectral height. (A) Overnight phosphorylation markedly decreases the ANS fluorescence of full-length CDC37 (NMC 14h), whereas isolated N and MC-domains show a low and phosphorylation-insensitive fluorescence. The sum of N and MC-domains fluorescence is added for comparison and molar equivalent but is reduced in intensity. Low ANS fluorescence was observed for CDC37 lacking the first 19 amino acids (Δ1−19). Data are normalized to the fluorescence of untreated NMC. For all ANS assays, excitation occurred at 380 nm and CDC37 concentrations were normalized based on the OD280 estimated protein concentration. (B) The N-domain in isolation is prone to rapid aggregation in PBS. N and NMC constructs were compared by high-speed centrifugation and western blot analysis for their solubility after dialysis and comparable storage. (C) Comparative characterization of the purified N-domain by CD. The concentration-corrected spectrum was obtained immediately after purification and spin column desalting.
exhibits a high tendency to aggregate post purification (Figure 2.4B). In fact, all N-domain-related studies were carried out immediately after purification with spin column desalting replacing dialysis in order to obtain reproducible results. To address this apparent contradiction between the obtained ANS signal and protein properties, we determined the CD spectrum of the N-domain. Consistent with the low ANS signal, this indicated α-helical structure (Figure 2.4C). The intensity of the concentration adjusted CD spectrum is proportional to the size of the N-domain compared to full-length CDC37, suggesting that the freshly isolated N-domain does not display a lack of secondary structure.

2.4.3 The chemical characteristics of serine are needed to retain CDC37 in its less compact state

Phosphorylation at serine or threonine can, in many cases, be mimicked by substitutions with glutamic or aspartic acid. We therefore investigated the impact of these substitutions on the ANS signal (Figure 2.5). Indeed, both aspartic and glutamic acid substitutions result in ANS binding that resembles the phosphorylated state. However, the same is true for substitution with alanine. These data suggest that the

Figure 2.5. Increase in compactness requires the removal of Ser-like properties. The ANS assay shows a marked decrease in fluorescence that is comparable to the phosphorylation of wild-type CDC37 (WT (14 h)) when serine 13 is mutated to alanine, aspartic acid, or glutamic acid. The biochemically more conserved mutant S13C retains the fluorescence pattern of wild-type CDC37.
observed transition is not driven by the introduction of the phosphate group but by the loss of serine.

By comparison, the substitution of serine with cysteine creates a wild-type-like signal. The same data were obtained for substitution with threonine (data not shown). However, the substitution by threonine is less straightforward in its interpretation. Its properties resemble those of the unphosphorylated state, but the potential presence of phosphorylation by CK2 cannot be ruled out definitively with the tools available. Overall, substitutions studies indicate that the loss of the side chain properties of Ser13, not the introduction of a phosphate group, causes the observed transitions in the structure of CDC37.

2.4.4 The N-terminal segment of CDC37 may be structurally adaptable

The above studies show substantial structural rearrangements upon phosphorylation, resulting in a more compact end state. Furthermore, this appears to involve relatively small net changes in actual secondary structure components. Similar phosphorylation-driven transitions between states of increased compactness have been described for intrinsically disordered proteins (IDP). We therefore analyzed the CDC37 primary sequence with the PONDR suite of algorithms (Predictor of Naturally Disordered Regions). Figure 2.6A shows the superimposition of the four individual algorithms that were combined in this analysis. The consensus of the four predictions assigns a high probability of disorder to two regions of CDC37: the M to C domain transition (approximately residues 250–325) and most of the N-domain (approximately residues 25–170). Yet, both regions are assigned a high α-helical content in secondary structure
prediction (Figure 2.6B). The secondary structure prediction matches available crystallographic data, where available, including the exceptionally long α helix found in the M section. With the exception of the VL3 algorithm (which evaluates a 30 amino acid window), the remaining algorithms show a high agreement in predicting a tightly defined area of disorder at the extreme N-terminus. This small segment of predicted disorder is centered on Ser13.

2.4.5 A phosphopeptide derived from the N-terminal tail of CDC37 induces the structural changes observed after phosphorylation

Since the phosphorylation of the N-terminus correlates with the observed decreases in ANS binding and this segment is set apart as a distinct entity in the prediction of intrinsic disorder, we generated two peptides that represent residues 1–19 of the N-terminus. The
peptides differed by carrying either serine or phosphoserine at position 13. At 25 °C, the addition of both peptides induces a moderate decrease in the ANS signal (Figure 2.7A, panel II). Although this decrease appeared to be more pronounced for the phospho-Ser13 peptide, differences were not large enough to be conclusive. We therefore tested whether the addition of peptides in trans influences the compactness of CDC37 under thermal stress. To this end, we measured ANS fluorescence at an elevated temperature of 50 °C. As a proof of principle for this assay, we first used dihydrofolate reductase (DHFR) and its stabilization by methotrexate (MTX) (Figure 2.7A, panel I). The binding of MTX to DHFR is well known to exert a strong stabilizing force, strong enough to block the unfolding of DHFR fusion constructs in the context of post-translational mitochondrial import. In the absence of a destabilizing temperature challenge, DHFR receives a measurable, but relatively small, stabilization from MTX. The stabilizing effect of MTX is significantly enhanced at 50 °C. While by itself nonphysiological, this stabilization at elevated temperature provides a much better representation of the strong barrier to unfolding that methotrexate binding introduces under physiological conditions. CDC37-derived peptide in isolation or when added to DHFR did not alter the ANS signal. When the same temperature challenge is applied to CDC37, a stabilization by the phosphopeptide becomes readily apparent, whereas the stabilization by the unphosphorylated peptide remains small. These observations suggest that the impact of phosphorylation at Ser13 induces interactions of the peptide with the remainder of the protein that convey a more compact structure that is more resistant to structural changes which expose ANS binding sites. This stabilization is readily evident in the phosphorylated species of CDC37 (Figure 2.7A, panel III), which is effectively
undisturbed by the increase in temperature. The deletion of the N-terminal tail (Figure 2.7A, panel IV) does generate an intermittent state that is only partially responsive to peptide addition. More importantly, it does not discriminate with regard to the nature of the peptide.

Figure 2.7. Phosphorylation at Ser13 or the addition of a (phospho-Ser13) peptide in trans stabilizes CDC37. (A) Panel I: Validation of ANS assay at elevated temperatures using DHFR and its stabilization by methotrexate at room temperature and 50 °C. Methotrexate, but not the pSer13 peptide (pSer), stabilizes DHFR upon temperature elevation. Panel II: ANS binding to unphosphorylated CDC37 rises at elevated temperature. A clearly discernible stabilization by the (phospho-Ser13) peptide (pSer) occurs at 50 °C. All peptides were added at a 1:1 molar ratio. Panel III: Compared to that of unphosphorylated CDC37, phosphorylated CDC37 has greatly reduced ANS binding at room temperature and is resistant to temperature increases. Quantitatively, the impact of the unphosphorylated peptide is small, but it is the only scenario in which peptide addition caused an increase in ANS fluorescence. Panel IV: The Δ1–19 truncation construct shows comparable starting fluorescence to that of pCDC37 but lacks its thermal stability. Modest stabilization occurs for both peptides, regardless of their phosphorylation state. (B) Phosphorylated serine 13 is partially shielded from phosphatases in a temperature-dependent manner. (Left) Phosphorylation at Ser13 by CK2 is close to maximal after 3 h. (Right) The access of pSer13 to a high concentration (0.1 U/μL) of alkaline phosphatase for 1 h is incomplete despite a large access of phosphatase. Dephosphorylation efficiency increases sharply above room temperature. Room-temperature dephosphorylation is complete when CDC37 is heat-denatured by SDS or Triton X-100 (TX), at the indicated concentrations, prior to a 10-fold dilution into phosphatase reaction buffer.
A remarkable feature of the phosphorylation at Ser13 is the physiological requirement for a specific phosphatase to carry out dephosphorylation in the context of the client and Hsp90 containing complex. Outside of this structural context, in vitro dephosphorylation of pCDC37 can be achieved with aggressive alkaline phosphatase treatment. We therefore wanted to evaluate whether the structural transitions that we observed alter the ability of a generic phosphatase to gain access to Ser13 (Figure 2.7B). A time course of phosphorylation by CK2 shows that close to the maximum attainable phosphorylation can be achieved within 3 h at room temperature. Following inhibition with 4, 5, 6, 7-tetrabromobenzotriazole (TBB, a CK2-specific inhibitor), treatment with a high concentration of alkaline phosphatase (CIP) ensures that enzyme levels are not a limiting factor. Under those aggressive dephosphorylation conditions, phosphorylation at Ser13 can be reversed, but it exhibits a very pronounced temperature dependence. At low temperatures, dephosphorylation remains partial despite large phosphatase access. Dephosphorylation efficiency increases rapidly above room temperature. Enzyme levels or the enzyme’s temperature dependence are not the limiting factors at and below room temperature, as CDC37 that was subjected to structure disrupting treatments prior to dilution into dephosphorylation buffer is efficiently dephosphorylated, even at room temperature.

2.5 Discussion

As a cochaperone that delivers a broad range of kinase clients to Hsp90, CDC37 is postulated to undergo a complex series of transitions in protein interactions. Phosphorylation at the N-terminal tail by CK2 is critical in the overall function of CDC37 and its interaction with the Hsp90 machinery, but it is not clear what the structure of the
N-terminal tail, and indeed the entire N-terminal domain, is and how phosphorylation alters function. In this study, we show that upon phosphorylation CDC37 shows a small but reproducible increase in secondary structure that is accompanied by a much more pronounced change in ANS binding. This suggests a more compact end state that is experimentally characterized by a remarkably high resistance to opening up at elevated temperatures. Indeed, on the basis of this readout, the stabilization that occurs as a result of phosphorylation is comparable to the stabilization that DHFR receives from binding methotrexate. For CDC37 molecules that initially folded with an intact N-terminal tail, this two-state transition can be induced in trans by a synthetic (phospho-Ser13) peptide representing the first 19 amino acids. The ability of the phospho- and nonphosphopeptide to compete in trans against existing N-terminal tails in the opposite phosphorylation state suggests a hierarchy in interactions. The higher stability of the phosphorylated state is evident from the ability of the (phospho-Ser13) peptide to achieve thermal stabilization of CDC37 in trans, even in the presence of a competing unphosphorylated tail in cis. The opposite scenario of the addition of unphosphorylated peptide to phosphorylated CDC37 is quantitatively too small to be statistically significant. However, it is qualitatively the only instance in our studies in which the addition of peptide ever increased the ANS signal.

Figure 2.8 presents a conceptual model for the different interconvertible states of CDC37 in terms of the energy landscape and rationale for the observed changes in ANS signal and secondary structure. The more compact mode observed for the phosphorylated state is also the default for the isolated N and MC-domains, and the generation of a more ANS accessible ground state requires both the interaction of the N and MC domains as well as
the regulating presence of the unphosphorylated N-terminal tail. This model of interaction between the N-terminal tail and the MC domain is also consistent with reports that tyrosine phosphorylation at positions 4 and 298 disrupts the interaction between CDC37 and client. The incorporation of the tail segment in Figure 2.8 reflects this concept of a dependency on N–MC interactions, but the specific placement of the tail interaction site is arbitrary, and the tail could alternatively influence the N–MC interaction allosterically.

Figure 2.8. Visual presentation of the hypothesis for CDC37 structural transitions, derived from current data. The model is not intended to describe specific structural features but, instead, to provide a conceptual framework that best reconciles the current data. Detailed discussion is provided in the text. Note that current data cannot distinguish between a direct tail interaction in the N–MC interface (depicted here) and potential allosteric regulation across domains. Gray areas represent amphiphilic to hydrophobic segments in the interface and tail sequence as well as their packing and degree of secondary structure. To maintain a state in which secondary structure elements remain accessible to ANS requires stabilizing interactions, specifically interactions that rely on the biochemical properties of serine 13. The loss of these properties by phosphorylation (or biochemically divergent mutation) allows for additional, strong interactions of the tail peptide that are accompanied by ANS (green) exclusion and increase in secondary structure. This equilibrium is reflected in the relative competition strength of the respective peptides in trans (indicated by arrows). Complete absence of the tail during folding (yellow dashed energy landscape) results in irreversible and incorrect interactions when the N and MC-domains are held in close proximity. The presence of the tail segment (purple landscape) steers the folding process away from this trapped and largely irreversible state. Maximal ANS binding (green) occurs in state B when binding-suitable secondary structure elements are both present (absent in A) and accessible (reduced in C). The peptide coloring scheme (amplified below) reflects the very pronounced separation into an amphiphilic to hydrophobic N-terminus followed by an exceptionally acidic segment.
The properties of the N-terminal deletion construct (CDC37 Δ1−19) suggest that it has assumed a state that is outside of the framework of controlled transitions that we see for phosphorylated and unphosphorylated CDC37. On the basis of ANS assays, it appears very compact, and the overall protein properties are consistent with this assumption. However, this stands in contrast to the loss in secondary structure components based on CD measurements and its inability to discriminate between the addition of phosphorylated and unphosphorylated tail peptides added in trans (Figure 2.7, panel IV). The apparently contradictory ANS signal may reflect the fact that reduced ANS binding, in most cases, reflects an increase in compactness but can also be the consequence of a loss of secondary structure elements that serve as binding pockets. In the case of CDC37 Δ1−19, both may apply. This would suggest an additional function for the N-terminal tail, besides that of a reversible switch for CDC37 conformations. The more open state represented by the unphosphorylated state may be prone to collapse in a way that involves inappropriate N–MC interactions, unless this is actively being blocked by the tail segment. This assumption is consistent with our observation that both the phosphorylated and unphosphorylated states form distinct interactions with the remainder of the protein. The state that is obtained when CDC37 folds in the complete absence of the N-terminus would, therefore, represent a soluble and stable, but nevertheless unproductive, dead end in the folding landscape of CDC37. In this context, the N-terminal tail may serve as an intramolecular chaperone.

The observed changes in ANS binding are far more extensive than those seen by CD spectroscopy. The latter only involve the transition of a few amino acids (estimated 10) from a random-coil to an α-helical conformation. Furthermore, a PSIPred secondary
structure prediction is in good agreement with experimental data for the M and C-domains as well as CD data for the isolated N-domain in predicting a large proportion of α-helical content, including for most of the N-domain. The above observations can be reconciled with the assumption that the observed structural transitions involve significant changes in the packing behavior of already formed α helices. Several predictors of disorder assign a high aggregate probability of IDP properties to portions of the MC-domains. Moreover, except for the extreme N-terminal tail, the entire N-domain receives high scores that are either contiguous or interrupted by short segments of predicted order, depending on the algorithm. With respect to alternative concepts on IDPs, our data for CDC37 are, therefore, more in line with preformed structural elements whose interactions are in a state of flux. This would also explain why the N-terminal domain shows a high degree of α-helical content yet is far more prone than any other CDC37 segment or mutant to aggregate in solution over a short period of time. Such a property to readily reorganize structural elements may contribute to the ability of CDC37, and specifically it is N-domain, to recognize a broad range of kinase clients. The N-terminal tail, in either phosphorylation state, would serve to maintain two interaction capable and interconvertible states instead of the state observed for CDC37 Δ1–19. This model is consistent with earlier studies that assigned functional contributions to the tail region in client binding and maturation.21

Protein phosphorylation has been observed before as a functional switch in IDPs.39–42 Furthermore, a preponderance of acidic residues has been identified as favoring a disordered state.103 However, studies on the impact of further phosphorylation, especially at the N-terminus of proteins, have come to divergent conclusions.104,105 A striking
feature of the mechanism by which the N-terminal tail segment regulates CDC37 is that the introduction of the phosphate group is not causal for the observed transitions. Transitions occur due to the loss of serine-like properties at position 13. Cysteine, and most likely threonine, are functionally neutral replacements that retain the ability to keep CDC37 in the more open state. At the same time, the phosphorylated state is not characterized solely by the loss of serine-dependent interactions but establishes a different set of interactions. Figure 2.8 incorporates these observations by assigning serine-dependent interactions a lower degree of stability, whereas phosphorylation, or other non-serine-like replacements, remove this barrier and allow segments of the tail region, especially its very amphiphilic to hydrophobic first 10 amino acids, to engage the remainder of the protein. The number of amino acids involved and the experimental CD data would fit a model in which this extreme N-terminus assumes α-helical character. However, if the most N-terminal amino acids are, in fact, induced to form an α helix, then it would be strictly dependent on stabilizing interactions with its immediate surroundings. Neither peptide in isolation (even with considerable addition of stabilizers) forms an α helix in solution (data not shown).

In most cases, phosphorylation is a trigger that in some form or another results in the transmission of a signal, with few but very prominent exceptions in metabolic regulation. Even in the case of IDP-based regulation, phosphorylation is generally regarded as being the causal modification. The resulting view of molecular processes is effectively kinase- and phosphorylation centric. Although considered to be critical, phosphatases are regarded as a means to turn off a signal initiated by phosphorylation, even if this signal involves altering the structural state of an IDP-like domain. A hallmark of most of the
above reactions is the involvement of highly specific kinases and phosphorylation events that can be reversed by either generic phosphatases, or more often, class specific phosphatases with a range of clients. In addition, the phosphorylated state usually comprises a small portion of the total protein substrate pool. For CDC37, the inverse is true in all cases. Dephosphorylation occurs in the CDC37–Hsp90 complex and selectively requires PP5 phosphatase to join the complex. In the context of the Hsp90 complex, pSer13 is not sensitive to the attack of generic phosphatases. By contrast, phosphorylation occurs by a protein kinase with broad substrate specificity and constitutively high activity, and the phosphorylated species comprises most of the cellular CDC37. Hence, phosphoserine serves as a structural building block in the ground state of wild-type CDC37, and selective dephosphorylation provides a stability switch that is based on the specific introduction of a nonphosphorylated side-chain with serine-like properties.

What are the possible implications of this stability switch? An estimate of the relative abundance of the phosphorylated state of endogenous CDC37 in MCF7 cells revealed that it is effectively the default state, with a minimum of 70–80% of CDC37 or CDC37 complexes being phosphorylated at Ser13. This may, in part, reflect the high activity of CK2. Assuming a dimeric state, the approach taken would potentially overestimate the degree of phosphorylation if a semi-phosphorylated state is abundant. However, such an asymmetric state would also have significant functional consequences and, based on the micromolar estimates for dimer stability, is unlikely to be recovered at high yield under stringent immunoprecipitation conditions, let alone 3-fold sequential immunodepletion. Whether phosphorylation does modulate dimerization is not clear from existing and
current data, although earlier studies have implicated dephosphorylation in the
dissociation of CDC37 from the client Hsp90 complex, which is preceded by the
dissociation of the CDC37 dimer.\textsuperscript{3, 30} Although our current data cannot distinguish direct
interactions of the tail segment with the N–MC interface from allosteric interactions, the
consequences of tail phosphorylation on the packing of CDC37 require the interaction of
the N and MC-domains. This would couple changes in the client-binding N-domain to the
dimerization and Hsp90 binding MC-domains. Our observation that the exact nature by
which Ser13 is altered is not relevant for the internal transitions of CDC37 may no longer
apply at later stages of complex formation. Instead, interactions outside the CDC37
monomer may make direct use of the properties of the phosphate group. The
phosphorylation state of CDC37 as it is released from these complexes may well impact
its stability. Together with the abundance of CK2, this may contribute to the low
abundance of the unphosphorylated state in a cellular setting. Previous studies had
already highlighted the fact that potential phosphomimetics and noncharged replacements
of serine alike generate similar phenotypes, both of which are deficient compared to the
actual phosphorylation of Ser13.\textsuperscript{30, 33} In a cellular setting with low CDC37 levels and
high levels of Hsp90, isolated CDC37– client complexes are very short-lived compared
to the more stable ternary complex involving Hsp90. It is, therefore, difficult to
differentiate deficiencies in the processing of the ternary complex from a reduced ability
of CDC37 to initially engage client. This may explain some of the divergent data on the
impact of mutations in the N-terminal tail of CDC37 on its function. The specific
contributions of the phosphorylated tail segment of CDC37 are likely to change as the
nature of protein complexes is being rearranged during client maturation. While the
current data are clearly limited in their ability to outline the nature of specific complexes, they provide new insight into dynamic transitions within the CDC37 cochaperone as it positions itself to engage client, and it provides an expanded and testable hypothesis of its mode of operation.
Chapter 3 The kinase specific cochaperone CDC37 functions as a structural defect discriminator in addition to kinase sorting

3.1 Summary

ERBB3 has a highly impaired kinase with only 0.1% activity of EGFR, due to absence of a glutamate in the αC helix and replacement of the HRD catalytic base aspartate with asparagine. The K723M mutation of ERBB3 abrogates the residual kinase activity and introduces subtle structural perturbation to the kinase domain. In stable cell lines expressing either the WT or K723M ERBB3, K723M has similar mRNA level and protein stability as WT, but demonstrates a significantly reduced steady state protein level. In transiently transfected lines, the cell surface fraction of K723M is stable while its total protein undergoes extensive degradation. We examined the chaperoning activity at the ER associated with the K723M mutant. CDC37 and Hsp90 form tight associations with K723M which potentially leads to ERAD. The phosphoserine mutants of CDC37 were found deficient in recognizing the structural perturbation at the kinase domain of K723M. This study presents CDC37 as a vital molecule in the structural proofreading of kinases.

3.2 Background remarks

The ERBB family of receptor tyrosine kinase (RTKs) consists of four members: EGFR/ERBB1, ERBB2/Neu/HER2, ERBB3/HER3, and ERBB4/HER4. Abnormal ERBB signaling due to elevated receptor level, mutation or autocrine stimulation has been implicated in a wide variety of solid tumors with the ERBB2 gene being amplified in 20-30% of metastatic breast lesions. Patients show initial response by receiving ERBB targeted antibody or small molecular inhibitor therapies, but longer term clinical studies
also indicated a high rate of relapse and resistance through diverse mechanisms including elevated ERBB3 activity. The normal actions of ERBB signaling are critical to maintain heart and cardiovascular development and functionalities.

A large portion of critical mammalian kinases require interactions with CDC37 and Hsp90, and this interaction can reside in the nascent state during the process of maturation alone or in both the mature and nascent state of the protein, possibly in qualitatively different association states. Recent studies in our lab showed that unlike ERBB2, the interactions of CDC37 as well as Hsp90 with wild type ERBB3 reside exclusively in the nascent state. Hence the study of chaperone interactions with ERBB3 provides an experimental route to the selective study of interactions in the nascent state and therefore to the study of structural proofreading of ERBB receptors.

For a long period, ERBB3 RTK was considered as a dead kinase lacking several catalytically important residues and serving mainly as an allostERIC regulator of its heterodimer partners. Fundamental biochemical studies recently revealed that ERBB3 binds ATP with a KD of ~ 1.1 µM and clustered ERBB3 on lipid vesicle is able to carry out autophosphorylation in trans. Preactivated and purified ERBB3 is capable of phosphorylating peptide substrates. The mutation K723M at the kinase domain completely abolishes ATP binding and kinase activity. Furthermore, the K723M mutation leads to much faster diffusion of ERBB3 on cell surface compared to that of wild type. Possible explanations include certain structural change of the kinase domain that affects N-lob mediated receptor interactions.
For many kinases, initial folding and the subsequent maturation require complex chaperone machinery. Following the formation of the very nascent conformation by disulfide formation and hydrophobicity, early folding assistance is carried out by Hsp40/70 and their accessory cochaperone complexes that mostly work on the exposed hydrophobic area. With the completion of early folding, kinases conveying certain kinase type feathers undergo extensive late folding with the involvement of comprehensive Hsp90 centered chaperone/cochaperone complex. CDC37 is known as the universal kinase specific cochaperone that works as a scaffold that first recognizes kinase substrates (referred as clients) and hands over to Hsp90. The coordination of successive cochaperone entry/exit, transition between various complexes of changing composition and even signaling events like phosphorylation/dephosphorylation constitutes the late folding as well as quality control (see review). Since CDC37 is the first molecule that identifies a nascent conformation as a kinase client, this interaction could be regarded not only as folding or maturation assistance but also quality control. Failure of a client to successfully pass through the successive arrangements may result in its degradation. As demonstrated for mutations in CFTR (cystic fibrosis transmembrane conductance regulator), severe functional deficiencies may arise due to relatively minor alterations in client structure and highly stringent quality control.

As a scaffold, CDC37 recruits Hsp90 through its M-domain and interacts with clients via its N-domain (with some exception like Vav3). For a subset of clients, CDC37 was indicated to process its clients independently of Hsp90. At the same time, CDC37 is not able to compensate for a loss of Hsp90 function, indicating distinct and non-redundant roles of CDC37 and Hsp90. The N terminal domain of CDC37 is
hypothesized to maintain structural flexibility in order to accommodate its wide spectrum of clients. The library of CDC37 clients covers over 350 proteins that involve almost exclusively ATP binding folds (mainly, but not exclusively, kinase).\(^{22}\) CDC37 also interacts with a limited number of non-kinase clients, including androgen receptor\(^{23}\), reverse transcriptase\(^{24}\), and the guanine nucleotide exchange factor Vav3\(^{25}\). Although overexpression of CDC37 occurs in limited cancers and at relatively modest levels, it has been shown to be a promising entry point for targeted therapy. Silencing CDC37 by siRNA or shRNA impairs the stability or production of many oncogenic kinases, such as ERBB2, cRAF, CDK4, CDK6 and AKT in various cancer lines.\(^{65-67}\)

The activity of CDC37 is proposed to be regulated by phosphorylation at its Ser 13 residue by CK2 (Casein Kinase 2).\(^{30}\) Mutations of Ser 13 site in yeast lead to severe growth deficiency and depletion of CDC37 clients.\(^{31, 32}\) However it is still not completely clear how phosphorylation regulates the action of CDC37 on the molecular level. A recently study in our lab demonstrated two distinct interactions between the N- and MC-domains of CDC37 stabilized by phosphorylated and unphosphorylated Ser 13.\(^{55}\) The ability of CDC37 in recognizing such a broad array of clients but rejecting closed related proteins might be associated with its intrinsic disorder property, but this needs further investigation. In this work, we focus on the actions of CDC37 as it confronts structural perturbation in its client ERBB3.

### 3.3 Materials and methods

**Constructs, cell lines and antibodies.** The set of CDC37 constructs (including WT, S13A, S13D and S13E) in vector pKH3 is a gift from Dr. Kerry Burnstein (University of
Miami). The ERBB3-Dendra-bio construct was generated by cloning ERBB3 cDNA into a pFlag-myc-CMV-19 vector. Sequences encoding Dendra, His tag and biotin acceptor peptide were added at the C-terminal subsequently. The K723M mutation was introduced by using QuikChange Lightning Kit of Agilent Technology. *E. coli* biotin ligase is expressed from construct pLXSN-Puro-BirA. Breast cancer line MCF7 was grown in RPMI medium supplemented with 10% FBS and 1% antibiotic cocktail and used for all the in vivo experiments. The constructs of ERBB3 in pFlag-myc-CMV-19 vector were cotransfected into MCF7 with a puromycin resistance plasmid. 0.2 µg/ml of puromycin was added in medium to maintain the stable cell lines (2 µg/ml for selection).

Western blot antibodies against GAPDH (8884), phospho-ERBB3 (4791) were purchased from Cell Signaling. ERBB2 (ab8054) and phosphor-ERBB2 (ab53290) antibodies were obtained from Abcam. Actin (sc-1615) and ERBB3 (sc-285) antibodies were purchased from Santa Cruz Biotechnology. Phosphotyrosine (05-321) antibody was bought from Millipore. His tag antibody (A00186) was a product of Genscript. Hsp90 antibody (ADI-SPA-840) and HA tag antibody (H9658) were purchased from Enzo Life Sciences and Sigma respectively.

**Quantitative PCR for relative mRNA level determination.** The stable or transient recombinant ERBB3 expressing cells were seeded in six well plates. Two wells of cells (~ 1 million) were combined for each sample. RNeasy mini kit from Qiagen was used for the extraction of total RNA. Reverse transcription was carried out with Random Hexamers and MMLV enzyme from Epicentre. Following the synthesis of cDNA, real time quantitative PCR was done with Sybr Green Fastmix solution of Quanta on a Roche light cycler.
**Immunoprecipitation.** Cells were grown on 6 well plates and harvested in mild lysis buffer (20 mM Tris, 137 mM NaCl, 1% Triton X-100, 10% Glycerol, 5 mM EDTA) containing 1 mM sodium orthovanadate and 1 mM PMSF. Lysate was further treated by passing through 26G X 3/8 needle to further disrupt cell membrane. For membrane proteins, lysate was incubated on a 37 °C heat block to effectively dissolve bounded membrane debris. The soluble fraction was separated by centrifugation and mixed with 15 µL of protein A/G beads (sc-2003 from Santa Cruz Biotechnology) and 4 µL of HA or His antibody. After one hour mixing/incubation in room temperature on a rotator, beads were washed with mild lysis buffer, boiled with the presence of 1X SDS sample buffer containing 15 mg/ml DTT and subjected to western blots.

**Time course transient expression of recombinant ERBB3.** MCF7 was seeded on 6 well plates, and transfected with either WT or K723M ERBB3-Dendra-bio constructs (0.5 µg/well). Dendra is a green-to-red photoswitchable fluorescent protein that exhibits fast maturation and bright fluorescence both before and after photoswitching. At its non-switched state, Dendra has similar fluorescent property as GFP. At various time points after transfection, the Dendra expression of both constructs was measured by taking fluorescent microscopy images as an indicator for ERBB3 expression. Fluorescence intensity of individual cell was quantified to generate plots of fluorescent distribution (negative cells were neglected). Following the microscopy, cells were lysed in mild lysis buffer and subjected to western blot or fluorescent spectrometry.

**Neutravidin pull down.** The bio tagged ERBB3 constructs were cotransfected with CDC37 and BirA constructs (0.5 µg of plasmid for each construct per well). *E. coli* BirA recognizes the biotin acceptor peptide at the C terminal tail of ERBB3 and carries out the
biotinylation in vivo utilizing the biotin present in the medium. 48 hours after the transfection, cells were lysed and further treated in the same way as an ordinary immunoprecipitation with the exception of usage of antibody and protein A/G beads. Supernatant from centrifugation was incubated with 30 µL of neutravidin agarose resin (29201 from thermo scientific) in room temperature on a rotator.

**siRNA and CDC37 knock down.** siRNA of CDC37 was purchased from Dharmacon (L-003231-00) as a mixture. Transient knock down of CDC37 was performed by transfecting MCF7 with 0.05 pmol siRNA or scramble per well (in 6 well plates). Lipofectamine 2000 from Life Technologies was used for the transfection of both siRNAs and plasmids.

### 3.4 Results

#### 3.4.1 The K723M mutant is extensively degraded, most probably in the ER under high translational loading

To study the role of CDC37 in the folding and maturation of defective clients, a kinase with distortions but that still largely folds well and maintains structural integrity is needed. Instead of newly designing mutations for suitable CDC37 clients and confirming their folding behavior, we adopted the well characterized K723M mutation for ERBB3. ERBB3 is a client of CDC37.\(^{55}\) Lys 723 is part of the ATP binding pocket of ERBB3 kinase domain and an amino acid conserved in all tyrosine kinases.\(^{56}\) Despite the potential structural perturbation, the K723M mutant folds and expresses in insect cells.\(^{57}\) Both K723M and its equivalent K721M for EGFR were reported to express on cell surface and respond to ligand stimulation.\(^{58,59}\) On the basis of these facts, K723M serves as a qualified kinase for our study.
To characterize the expression property of K723M, MCF7 was cotransfected with ERBB3-Dendra-bio constructs and a puromycin resistant plasmid. Stable lines were established by high puromycin (2 µg/ml) medium selection and maintained in moderate puromycin (0.2 µg/ml) medium. Comparable amount of recombinant ERBB3 mRNA were found in the WT and K723M lines (Figure 3.1A), indicating that both constructs were equally incorporated into the stable cell lines and subjected to similar transcription effectiveness. While both lines demonstrate higher ERBB3 levels than parental MCF7, K723M shows significantly reduced steady level of ERBB3 as compared to WT (Figure 3.1B). The translational efficiency of these two constructs should be comparable since they are identical with the exception of only one codon. The mismatch in the protein and mRNA levels can be caused by either difference in protein stability at mature state or differential folding/maturation. We employed cycloheximide chase assay to evaluate protein stability of WT and K723M, which showed similar half life of ERBB3 for the two lines (Figure 3.1C). These findings suggest similar molecular stability for ERBB3s harboring the K723M mutation and WT as they surpass

![Figure 3.1](image-url)  
**Figure 3.1.** Characterizing of the K723 mutant of EBB3 in stable line. (A) RT-PCR and (B) western blots for the transcription and protein levels of ERBB3 in the WT and K723M lines. The K723M cells had comparable amount of recombinant ERBB3 mRNA as the WT cells, but was significantly lower in protein level. (C) Cycloheximide chase assays showed similar half life (~ 1.5 hour) of total ERBB3 for WT and K723M lines.
the stages of folding/maturation, and a potential higher overall degradation for K723M at the stages of folding/maturation.

3.4.2 Transiently expressed K723M is to a large degree degraded prior to ER/Golgi export

The minimal subpopulation of ERBB3 receptors residing in folding and maturation stages restricts our investigation for the molecular events at these early times in the stable cell line. To overcome this limit, we deliberately enlarged the subpopulation at those stages by transient transfection. Fluorescent microscopy study of GFP tagged ERBB3 showed the majority built up in the ER area and the cell surface fraction represents a very small portion. When transformed with the same amount of plasmid DNA, the WT and K723M constructs deliver comparable ERBB3 mRNA levels (Figure 3.2A). We observed no obvious turn over for WT in a cycloheximide chase assay for total protein (Figure

![Figure 3.2](image.png)

Figure 3.2. Characterizing of the K723 mutant of EBB3 in transient setting. (A) RT-PCR and (B) western blots for the transcription and half life for transient WT and K723M lines. (A) Transiently transfected WT and K723M cells had comparable recombinant ERBB3 mRNA. (B) Compared to WT, K723M showed slightly lower ERBB3 that underwent degradation with a half life of ~ 2.5 hour, whereas WT was completely stable (Up panel: total protein). Cells were surface biotinylated to evaluate the half life of the cell surface localized ERBB3. The K723M line had stable cell surface ERBB3, while the WT line had a half life of ~2.5 hour (Bottom panel: cell surface). Notably, the cell surface fraction of K723M was significantly smaller than that of WT.

3.2B up panel), which is in line with the fact that the surface subpopulation, which undergoes regular receptor degradation pathway, only constitutes a small fraction of the
total protein after switching to the transient transfection setting. However, the K723M mutant starts with a slightly lower level and obtains a half life of ~2.5 hours in the same condition (Figure 3.2B up panel). In transient transfection ERBB3, the ER residing subpopulation of receptor is dominant and overwhelms the cell surface subpopulation. The above data implies with a more extensive degradation for K723M at its early stage.

The stable lines were cell surface biotinylated to determine the half life of ERBB3 (Figure 3.2B bottom panel). While WT shows a half life over two hours (regardless of the 0.5 hour bump), K723M is stable on cell surface. The facts that transient cells have the majority of ERBB3 residing at the stage before export from the ER, and K723M is stable on cell surface but fast degraded in total level again suggest extensive degradation of K723M at the ER. Notably, despite similar transiently expressed ERBB3 level in the two lines, K723M shows significant lower cell surface ERBB3 level, which might be the consequence of low success rate in ER/Golgi export.

3.4.3 K732M expresses slowly with a time delayed fashion in early stage of transient transfection

With all the available results pointing to a defect in folding/maturation for K723M, we expect to see some difference in the initial protein production between WT and K723M in transient transfections. Firstly the expression of K723M in early stage of transient transfection was monitored by western blots assay. Figure 3.3A is the western blots for the ERBB3 levels (indicated by His tag) for WT and K723 transfects at various time points post transfection. Figure 3.3B is the graphic representation of quantitative data for the western blots after quantification of band intensities in software Image Studio. The base line of gradient expression is given by the WT curve. Comparatively K723M shows
significantly slower accumulation at the first 40 hours and has a tendency of catch up at 48 hours.

One way to evaluate the folding efficacy of a given protein is fluorescent folding reporter assay. Dendra is a green-to-red photoswitchable fluorescent protein that exhibits fast maturation once folded. At its non-switched state, Dendra has similar fluorescent property as GFP. For the ERBB3-Dendra constructs, formation of the chromophore of Dendra depends on the coupled folding of the 5' ERBB3 receptor. As a result, emerging of fluorescence could serve as an indicator for the folding completion of the ERBB3

Figure 3.3. The expression of K723M is slow with a time delayed pattern at the first 48 hours of transfection. (A) Cell lysates of transiently transfected MCF-7 were collected at indicated time points and subjected to western blot analysis after normalization of protein concentration by BCA assay. K723M level was significantly lower at 24 and 40 hours, and had the tendency to catch up with WT over 48 hours. (B) A time delayed pattern of K723M expression is evidential in the plot of protein expression with quantitative values of (A). (C) Formation of the chromophore of Dendra depends on the folding of the whole protein. Thus, Dendra fluorescence could be used as a reporter for correct folding of ERBB3. The lysate samples for (A) were subjected to fluorescent measurement. The patterns of fluorescence increment for WT and K723M are similar to the protein levels in (B) suggesting WT and K723M receptors are folded proportionally the same.
receptor. Cell lysates of the two transient lines at the indicated time points were collected and subjected to fluorescent measurement in a fluorescent spectrometry (Figure 3.3C). Similar tendency of progression in fluorescence for both lines was observed as that of western blot in Figure 3.3B. This suggests that these two constructs have comparable folding/translation ratio and the degradation issue for K723M intervenes post this point.

As an alternative way to assess the initial ERBB3 production in transient transfections, we used fluorescent microscopy to quantify the emergence of Dendra fluorescence.

![Figure 3.4](image)

**Figure 3.4.** Population profile of fluorescence was collected by measuring the intensity per unit area of individual cell at the indicated time points and plotted (negative cells were neglected). K723M line showed a pronounced time delay in turning green (slower expansion of the area under curve toward right).

Population profile of fluorescence was collected by measuring the intensity per unit area of individual cell at the indicated time points and plotted in Figure 3.4 (negative cells were neglected). Consistently with the finding in Figure 3.3A+B, K723M shows a pronounced lag in turning green (slower expansion of the area under curve toward right).

### 3.4.4 K723M tightly associates with CDC37

The characterization of K723M in terms of stability at the cell surface and total level in both transient and stable setting points to extensive degradation of the mutant before export to cell membrane. The evident observation that K723M is expressed in a time delayed fashion at early stage of transfection also indicates a disadvantage of K723M in
expression. However, fluorescence reporter assay suggests no defect of K723M in initial folding. Thus we focus on the structural proofreading, the step after generic folding is achieved, of ERBB3 at the ER for any explanation. The structural proofreading of kinases heavily relies on the Hsp90-CDC37 complex. Although ERBB3 is an untypical kinase because of its lacking of several critical catalytic residues and weak, if any in vivo, kinase activity, it is still a client of CDC37-Hsp90 complex.\(^5\) In a transient cotransfection of ERBB3 and siRNA of CDC37, the expression of both WT and K723M ERBB3 are severely impacted by CDC37 knock down (Figure 3.5A). The mutation of K723M blocks the ATP binding and diminishes the residual kinase activity of ERBB3, but does not prevent ERBB3 from remaining a client of CDC37. The association status of

Figure 3.5. K723M tightly associates with CDC37. (A) In spite of the mutation at ATP binding pocket, K723M expression was severely impacted by CDC37 knock down, indicating it is still a client of CDC37. (B) Hsp90 and CDC37 associations of ERBB3 were evaluated in neutravidin pull down assay. Elevated Hsp90 and CDC37 interactions were found for K723M. (C) The elevated CDC37 association of K723M is heavily Hsp90 dependent as Geldanamycin treatment abolishes CDC37 interaction to a comparable level as that of WT.
ERBB3 with the chaperone complex was evaluated in neutravidin pull down assay where bio tagged and in vivo biotinylated ERBB3 was pulled down with neutravidin agarose beads. Despite its less protein level, K723M shows elevated interaction with CDC37 (Figure 3.5B). In the case of CFTR, tighter association of mutant client with Hsp90 complex is indicative for higher ERAD (ER association degradation).\textsuperscript{2} CDC37 is considered as a transit scaffold that exits the complex relatively fast for native and correct folded kinases, but apparently not for K723M in our study. Notably, Hsp90 association is also elevated for K723M. To closely examine the nature of these two increased association, we applied an Hsp90 inhibitor, Geldanamycin into the system. Addition of Geldanamycin completely abolishes the elevated CDC37 association (Figure 3.5C), indicating the tighter K723M-CDC37 association is Hsp90 dependent. CDC37 as the first molecule contacting with kinases in the quality control, may recognize the kinase domain of ERBB3, detect the conformation perturbation of the K723M mutation, convey this “error” information to the later complex and eventually leads to a frozen complex that is prepared for degradation.

3.4.5 Phosphoserine mutants of CDC37 lose the ability to recognize the structural perturbation of the K723M mutation on ERBB3
The molecular property and chaperoning activity of CDC37 are regulated by the phosphorylation at its Ser 13 site. It has been shown that Ser 13 mutations impact client recognition.\textsuperscript{33, 34} We wanted to know what the impact of Ser 13 mutations is in the ability of CDC37 to recognize the structural perturbation of K723M. A serial of neutravidin pull downs were carried with different combinations of ERBB3 and CDC37 (Figure 3.6A). For WT ERBB3, we did not observe any obvious variation in associated CDC37s including WT, S13A, S13E and S13C. Remarkably, all the mutants of CDC37 lost the
ability to form tighter interaction with the K723M mutant of ERBB3 (Figure 3.6A, third panel). This was affirmed in the reverse direction immunoprecipitation by HA antibody to pull down CDC37 (Figure 3.6B first panel). The K723M mutation elevates the interaction of Hsp90 with ERBB3 no matter which version of CDC37 presents (Figure 3.6A second panel). It is either because the mutants of CDC37 were defect in holding in the frozen complex but not in transmitting the error information to the later complex, or because Hsp90 can act through an alternative pathway without the involvement of CDC37. S13A but not the other mutants showed diminished Hsp90 interaction for some reason not known (Figure 3.6B second panel).

![Figure 3.6](image)

Figure 3.6. Mutations on Ser 13 site impair the ability of CDC37 in recognizing the structural perturbation of K723M. (A) Ser 13 mutations didn’t affect the basal interaction of CDC37 with WT ERBB3. WT was the only version of CDC37 that demonstrated an elevated association with Hsp90. Hsp90 also tightly bound with K723M, regardless of the CDC37 present. (B) CDC37 was immunoprecipitated with anti HA antibody and associated ERBB3 and Hsp90 were analyzed. Consistent with the result in (A), K723M ERBB3 and WT CDC37 was the only combination having high association.

### 3.5 Discussion

CDC37 works as a kinase recognition module that sorts out kinases and rejects any other type of proteins for the later process of structural proofreading. As the only component and property conserved across all classes of kinases and even non-kinase clients of
CDC37, the ATP binding pocket most probably serves as the determining structural element for newly folded kinases to be recognized by CDC37. Kinases that end up misfolded due to malfunctions of the Hsp40/70 machinery or inherent structural perturbation (such as mutations) are rejected by CDC37 and thus the whole structural proofreading, because of the missing ATP binding pocket resembling structures. These molecular events constitute the most fundamental role of CDC37 in the structural proofreading.

Unlike random mutations at the kinase domains that often results in unfolds, misfolds and fast destruction, the K723M mutation delivers ERBB3 receptors that express in both insect and mammalian cells and localize on cell surface as normal57-59 and thus stands for a fold with defects. Not only being still recognizable by CDC37, the ATP binding pocket of K723M forms extraordinarily tight interaction with CDC37. The ability of forming tight complex with well folded kinases with defects requires native serine at the 13 position. This represents the advanced function of CDC37 in the structural proofreading.

As the only kinase (or ATP fold) specific component in the structural proofreading, CDC37 should be able to discriminate the defect in a kinase fold independently from the rest of the machinery. However, its staying in the tighter triple complex relies on Hsp90 as Geldanamycin abolishes K723M-CDC37 interaction to the ground state.

Classically, the engagement of kinases with Hsp90 follows a restrict order that is bridged by CDC37. Figure 3.6A shows elevated Hsp90 association with K723M regardless of CDC37 status. This indicates the potential existence of CDC37 independent interaction of Hsp90 with kinases with high tendency of misfolding. We proposed a third pathway in the picture of the structural proofreading of kinases (Figure 3.7 grey path). The K723M
A mutant of ERBB3 has similar folding efficacy as that of WT, but results in more profound misfolds that present no clear ATP binding pocket structure. Hsp90 directly works on these misfolds and redirects to ERAD. Higher extent of hydrophobic exposure might be the driven force for this untypical interaction. For the well folded potion of K723M, the inherent defect coming from the mutation is recognized by CDC37 and the degradation is through the frozen triple complex (Figure 3.7 red path). Nevertheless, we cannot exclude other possibilities that do not involve the previous third path for the

Figure 3.7. Scheme of the branches in CDC37 initialized structural proofreading. Folded kinases are recognized by CDC37 and engaged in the triple complex. For correctly folded kinases, such as ERBB3, CDC37 leaves the complex. At certain later stage the kinase passes the quality control and gets released from the chaperone complex. For those folds with defects, such as mutations, the triple complex freezes and is directed to ERAD. The other class of folds that does not form kinase structures is classified as misfolds. This class engages with Hsp90 in a CDC37 independent manner and goes to ERAD.
observation in Figure 3.6 A. As an example, the mutants of CDC37 are deficient in staying in the triple complex.

Compared to Hsp90, CDC37 has narrowed clients that are mostly kinases. And many of them are critical kinases in cancer promoting pathways. This represents the advantage of targeting CDC37 over Hsp90 for cancer treatments. This study implies two possible functional interferences for CDC37. Firstly, inhibition of CDC37’s ability to recognize kinase leads to downregulation of its clients overall. Secondly, rescue of degraded kinases with structural defect could be accomplished by interference with CDC37’s ability to discriminate the structural defect in kinases. My work in chapter 5 is a practice for the first aspect.

The conformation of a newly folded kinase is critical for its CDC37 recognition. An approach to assess the conformational property of kinases will be extremely beneficial to the evaluation of folding efficacy and the mechanism studies of kinase recognition by CDC37 and thus facilitate my study. We participate in a collaborative project with Dr. James Wilson at the Department of Chemistry to develop fluorescent activation state specific (FAST) probes for ERBB receptor tyrosine kinases. While Dr. James Wilson’s lab contributes their expertise in fluorescent probes, chemical synthesis, purification and identification, we complement with the knowledge of ERBB inhibitors and cell based assays. Presented in Chapter 4 is a summary of selected publications of this work.
Chapter 4 Development of “turn on” fluorescence probes with potential applications in querying receptor kinase states

4.1 Summary

With the aim of developing optical tools to investigate ERBB populations and their state of activation, we have synthesized a fluorescent “turn-on” probe, DMAQ (Figure 4.1), targeting the ERBB ATP binding pocket. DMAQ combines both an optical reporting element and a pharmacophore with demonstrated affinity towards members of the ERBB family. Upon binding, probe emission increases due to the hydrophobic environment and restricted geometry of the ERBB2 kinase domain, facilitating the analysis of receptor states at low occupancy without the removal of unbound probes. Cellular ERBB2 autophosphorylation is inhibited with saturation kinetics that correlate with the increase in probe fluorescence.

With the accomplishment of fluorescent “turn-on” probe DMAQ, we continue to investigate the effect of conjugation length and auxochrome substitution at the fluorophore arm on the optical properties of a family of N-phenyl-4-aminoquinazoline probes that target the ATP-binding pocket of the ERBB family of receptor tyrosine kinases (Figure 4.2). Extension of the aromatic quinazoline core with fluorophore “arms” through substitution at the 6- position of the quinazoline core with phenyl, styryl, and phenylbutadienyl moieties was predicted by means of TD-DFT calculations to produce probes with tunable photoexcitation energies and excited states possessing charge-transfer character. Optical spectroscopy identified several synthesized probes that are nonemissive in aqueous solutions and exhibit emission enhancements in solvents of low polarity, suggesting good performance as turn-on fluorophores. Ligand induced ERBB2
phosphorylation assays demonstrate that despite chemical modification to the quinazoline core these probes still function as ERBB2 inhibitors in MCF7 cells. Two probes were found to exhibit ERBB2-induced fluorescence, demonstrating the utility of these probes as turn-on, fluorescent kinase inhibitors.

4.2 Background remarks

4.2.1 Fast probes and DMAQ

ERBB receptor kinases play a crucial role in normal development and cancer malignancies. A broad range of modifications creates receptor subpopulations with distinct functional properties in live cells. The readily accessible and commonly used surrogate to study the levels of activated ERBB is the measurement of tyrosine phosphorylated substrates, including the tyrosine phosphorylation of the receptors themselves. However, this approach cannot differentiate between contributions from high levels of marginally activated receptors and small populations of hyperactive receptors, a distinction with significant mechanistic and ultimately clinical relevance. In addition, “pseudoactivation” occurs when the balance of tyrosine phosphorylation and dephosphorylation is upset. This is for example the case when reactive oxygen species (ROS) inhibit cellular tyrosine phosphatases. As a consequence, elevated ROS can trigger a rapid “pseudoactivation” of ERBB receptors that rivals ligand stimulation based on phosphotyrosine levels, but with fundamentally different signaling properties and modes of signal propagation. Many aspects of this complex interplay are not readily accessible with current methodologies and new methods to obtain direct information on the state of the receptors under different cellular conditions would be very desirable.
As an important extension of the structural proofreading project, we collaborate with Dr. James Wilson to design and develop Fluorescent Activation State specific (FAST) probes based on the availability of inhibitors and ATP cocrystal structures of ERBB kinase domains. Activation state specific probes can be used to selectively query the nascent state when fluorescent resonance energy transfer (FRET) is being used in conjunction with fluorescent fusion constructs of cochaperones or, as used by us in, photo convertible Dendra2-fusions of the ERBB receptors. Photoconverted preexisting Dendra2 will have red fluorescence while newly emerging, nascent fusion proteins have GFP-like green fluorescence providing a nascent selective donor or acceptor for FRET. FAST probes can either be inherently fluorescent or become fluorescent in the context of their binding pocket giving rise to so called “Turn-On probes”. With these molecular tools, conformational properties of ERBB receptor kinases, in active state, inactive state

![Design strategy for a ‘turn-on’ fluorescent ligand: incorporation of a push–pull chromophore similar to DCS and an ERBB-targeting pharmacophore resembling 1, yields DMAQ, which is comparable in overall size to Gefitinib (an EGFR inhibitor).](image)

Figure 4.1. Design strategy for a ‘turn-on’ fluorescent ligand: incorporation of a push–pull chromophore similar to DCS and an ERBB-targeting pharmacophore resembling 1, yields DMAQ, which is comparable in overall size to Gefitinib (an EGFR inhibitor).
or ATP binding pocket unfolded state can be readily assessed in the context of CDC37 interaction.

As a first and critical step towards such probes, we have synthesized a molecule, 6-(4-dimethylaminostyryl)-N-benzylquinazolin-4-amine (DMAQ, Figure 4.1), which targets the ATP binding site of ERBB2 with desirable micromolar affinity while retaining the specificity of the 4-aminoquinazoline family of derivatives that constitute a large class of ERBB inhibitors with well-characterized pharmacology. N-phenyl derivatives such as Gefitinib or Erlotinib as well as N-benzyl derivatives such as 1 that exhibit micromolar to nanomolar affinities for ERBB members. In a general sense, DMAQ may be viewed as a fluorescent adenosine analog yet lacks the ribose unit found in other fluorescent adenosine mimics. In designing DMAQ, we considered the need to combine both an optical reporting element and a pharmacophore with demonstrated affinity towards members of the ERBB family. Incorporation of the optical reporter was achieved by extending the conjugation of the quinazoline core via a dimethylamino-substituted styryl arm. This modification is not expected to alter the binding mode of the quinazoline core based on the molecular orientation of several quinazoline-based inhibitors observed in ERBB kinase crystal structures. The electron withdrawing nature of the quinazoline functionality coupled opposite the electron rich styryl substituent creates a compact donor-p-acceptor system with attractive physical and optical properties similar to 4-N, N-dimethylamino-40-cyanostilbene (DCS). The inherent fluorescence of DMAQ avoids the need to couple an external fluorophore eliminating additional steric bulk of the fluorophore and tether. Synthesis of DMAQ was achieved in two steps via
condensation of benzyl amine with 4-chloro-6-iodoquinazoline followed by the palladium catalyzed coupling of N, N-dimethyl-4-vinylaniline.

### 4.2.2 Emission Tuning by altering conjugation length and auxochrome substituent

Alternatively we develop Fluorescent Activation STate specific (FAST) probes that are built upon the 4-aminoquinazoline scaffold, which is an established EGFR/ERBB pharmacophore; examples include Gefitinib or Erlotinib (Figure 4.2A,C), so-called type I inhibitors that preferentially binding the active kinase conformation, and Lapatinib (Figure 4.2B,D), a type II inhibitor targeting the inactive conformation. The preference for active and inactive conformations is addressed through substitution at the 4-amino position (Figure 4.2E).

While the quinazoline core conveys binding to the nucleotide pocket, but with limited specificity for ERBB-type receptors, the 4-amino aryl arm further increases specificity and contributes discrimination between activation states. This selectivity reflects

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**Figure 4.2.** Crystal structures of the EGFR ATP-binding pocket with (A) erlotinib (PDB ID: 1M17) and (B) Lapatinib (PDB ID: 1XKK) reveal the inhibitor binding modes. The arms at the 6-position of the quinazoline core (in blue; C, D) may be replaced by fluorophore arms without disturbing the key binding contacts. (E) General structure and substituent key for the synthesized fluorescent quinazolines.
increased access to the hydrophobic pocket adjacent to the nucleotide binding site in the inactive conformation. Together, the quinazoline core and N-aryl arm constitute the pharmacophore. Crystal structures of the kinase domain of EGFR with either Erlotinib (1M17)\textsuperscript{5} or Lapatinib (1XKK)\textsuperscript{6} (Figure 4.2A+B) show that while the pharmacophore arm is oriented deep in the binding pocket, the 6-position is amenable to chemical modification without perturbing the key conserved contacts of the binding pocket. The structure of Lapatinib demonstrates this point as the aromatic core is extended by the addition of a furan ring. Thus, a potential strategy for synthesizing fluorescent kinase inhibitors is to modify the 4-aminoquinazoline core with fluorophore arms (Figure 4.2E) at the 6-position. In principle, probes targeting specific kinases (i.e., IGF1R vs EGFR) or activation states could be encoded with unique optical outputs by tuning the excitation and emission energies through the fluorophore arm.

Herein, we investigate the effect of conjugation length and auxochrome substitution on the optical properties of a family of N-phenyl-4-aminoquinazoline probes. We find that extension of the π framework effectively lowers the excitation and emission energies, yielding fluorescent probes that compare favorably with other fluorescent adenosine analogues.\textsuperscript{113, 119-121} The introduction of strong electron donors or strong electron-withdrawing groups generates donor–acceptor systems that are highly sensitive to solvent polarity. As a result, several probes exhibit high fluorescence ON/OFF ratios, a feature that is key to their performance as self-reporting fluorescent kinase inhibitors. Despite the modifications to the quinazoline core, we also found that several of these probes inhibit ERBB2 phosphorylation in a live cell setting, demonstrating that binding to the ATP pocket and cell permeability are preserved. Overall, these fluorescent adenosine mimics
compare favorably with other nucleobase analogues as they possess tunable optical properties and high fluorescence turn-on ratios and compete effectively with the native substrate to inhibit tyrosine phosphorylation.

4.3 Materials and methods

General methods, reagents and instrumentation. The reagents and materials for synthesis were used as obtained from Sigma Aldrich and TCI America and used without further purification. ACS reagent grade solvents were obtained from EMD Chemicals. $^1$H NMR and $^{13}$C NMR spectra were recorded on Bruker 400 and 500 MHz spectrometers using tetramethylsilane (TMS) as the internal standard and chloroform-d (CDCl$_3$) as the solvent. Absorbance spectra were obtained on a Perkin-Elmer Lambda 35 UV-vis spectrometer. Fluorescence studies were performed on Perkin-Elmer LS55 and Jasco 6500 fluorescence spectrometers. For determination of $\varphi_{em}$ solutions were prepared to an optical density of 0.05, in order to minimize inner filter effects. Perylene in cyclohexane was used as a reference for quantum yields, which were calculated as previously reported.$^{122}$ Density function calculations were performed using Spartan (Wavefunction, Inc.) with the B3LYP basis set at the 6-31G* level.

Synthesis and characterization of 6-(4-dimethylaminostyryl)-N-benzylquinazolin-4-amine (DMAQ). To a clean, dry round bottom flask purged with argon was added 6-iodo-N-(phenylmethyl)-4-quinazolinamine 400 mg (1.1 mmol), tris(dibenzylideneacetone)dipalladium 70 mg (0.076 mmol), tri-terbutylphosphine 90 mg (0.44 mm, 0.4 equiv), cesium carbonate 500 mg (1.5 m mmol) followed by the addition of anhydrous dimethylformamide (10 mL). The resulting mixture was degassed with argon and
dimethylaminostyrene 160 mg (1.1 mmol) was added and the reaction was heated at
100 °C for 24 h to furnish the desired product as a yellow solid (yield: 12%, 51 mg) after
column purification (CH2Cl2 → 1:1 CH2Cl2:EtOAc).

**ERBB-dependant DMAQ fluorescence.** Spectrophotometric analysis was carried out on
a Jasco, FP6500 fluorescence spectrophotometer. Optimal excitation/emission conditions
were initially determined by 3D scans of matched Canertinib and non Canertinib treated
samples and subsequent subtraction of 3D spectra. All spectra were obtained in final 1x
PBS and intermittent dilutions of DMAQ were carried out in distilled water. Cell lysates
in PBS were diluted approximately 10-20 fold in most assays. For the measurement of
very dilute protein samples, such as soluble kinase domains in non-enriched lysates from
a single 3 cm well, spectra for the background fluorescence of DMAQ in PBS were
obtained in parallel and subtracted out. Effectively negligible background fluorescence
occurs for DMAQ in distilled water, but measurements in PBS and calculation of the net-
fluorescence was deemed more physiologically relevant. For these measurements, net
fluorescence was plotted as indicated. For all other measurements, relative fluorescence
is presented as measured without correction of free DMAQ fluorescence.

**Cell culture maintenance and lysate preparation:** MCF7 cells were maintained in
RPMI-1640 (10% fetal bovine serum, 5% CO2). At 70% confluency, MCF7 cells
carrying full-length and His-tagged-ERBB2 received RPMI with or without Canertinib
(300nM, CI-1033, LC Laboratories) followed by incubation under cell growth conditions
for 12 hours prior to lysis. Media was removed and washed cells were lysed in mild lysis
buffer (20mM Tris, 137mM NaCl, 1% Triton X-100, 10% glycerol, 5mM EDTA, 1mM
sodium orthovanadate, 1mM PMSF, 1X cocktail). Lysates were passed ten times through
a fine gauge needle and incubated for 5 min at 37°C. Cell debris was removed by centrifugation at 10,000g and supernatants were added to Ni-NTA column resin. After three rounds of washing, bound material was eluted for spectrophotometric analysis in PBS, 1mM EDTA. For post lysis Canertinib treatment, resin bound material was incubated with 300nM Canertinib for 1 hour at 37°C after one round of washes, followed by three additional washes and elution in PBS/EDTA. For the characterization of DMAQ binding to affinity enriched ERBB2, full length receptors were estimated by serial dilution and Western blot analysis of enriched material against purified standards. The determination of 10 pM receptors (or 6 x 1012 receptors per measurement) obtained by this approach is in good agreement with estimates based on the number of receptors per cell and the number of cells utilized for the analysis. Estimates derived for the affinity-enriched receptors were used to calculate the molar increase in DMAQ fluorescence. For the analysis of the soluble kinase domains in the context of whole cell lysate derived from less than one million cells, reliable molar estimates of kinase domain concentrations were not accessible.

**DMAQ fluorescence titration.** For the evaluation of DMAQ binding to the soluble ERBB2 kinase domain in the presence of other, soluble cellular kinases, MCF7 cells were transfected with either Cherry control or ERBB2 kinase domain Cherry fusion. For the creation of the ERBB2-KD-cherry fusion, the PCR product (primer 1: CGAGATCTATGAAGCGACGGCAGCAGAAGAT, primer 2: CGAAGCTTTTTACTGGGGTACCAGATACTCCT) was cloned into the BglII and HindIII sites of mCherry-C1 (Clontech). Stably transfected MCF7 cells were obtained by neomycin selection after lipofectamin mediated transfection. Cells were lysed through
sonication in PBS supplemented with protease inhibitor cocktail (HAT, Sigma) and insoluble material was cleared by centrifugation at 10,000xg. Lysates were normalized for protein content and processed without additional enrichment. Prior to lysis, matching cultures of Cherry controls or ERBB2 kinase domain-cherry fusions were treated with Canertinib as indicated above, free Canertinib was removed prior to lysis and equal recovery of fusion proteins was confirmed by the fluorescence of Cherry.

**Inhibition studies of DMAQ.** MCF7 carrying stably overexpressed and autoactivated ERBB2 (MCF7/B2) were seeded at 50,000 cells/well in a 12 well-plate in RPMI supplemented with 10% FBS. After 48h, media was supplemented for 12 hours with the indicated concentrations of DMAQ, Canertinib, or DMSO vehicle. Cells were lysed in hot SDS-PAGE sample buffer (75 mM Tris-HCl, 10% w/v glycerol, 3% w/v SDS, 2.4 mM bromophenol blue, with 52 mM dithiothreitol) and analyzed by Western blot analysis with antibodies against the C-terminus of ERBB2 (CB11, Biogenex) and site specific phosphorylation at Tyr1139 (pERBB2(Y1139), Epitomics). The inhibition of ERBB2 tyrosine phosphorylation was compared with that of the Ser/Thr kinase Akt in an MCF7 derivative cell line with constitutive PI3K dependent but ERBB2 uncoupled AKT activation. Inhibition studies were carried out as described above with the PI3K inhibitor NVP-BEZ235 (LC Laboratories) as a positive control. AKT activation was measured by western blotting for its feedback phosphorylation status on phospho serine 473 (pAKT(Ser473) and AKT, Cell Signaling Technologies).

**Inhibition of ligand-induced receptor activation.** MCF cells were seeded with equal quantity (200,000/well) in six-well plates. After 48 h, cells were pretreated with small molecule inhibitors of various concentrations for 1 h before induction by neuregulin
(NRGβ1, 20 nM, 15 min). Cell lysates were generated immediately by SDS lysis. Equal aliquots were subjected to SDS-PAGE and western blot analysis. ERBB2 phosphorylation was evaluated for Tyr1239 located close to the extreme cytoplasmic C-terminus of the receptor (validated by pan-TyrP detection (4G10)). The signal obtained for pTyr1239 relative to the ERBB2 receptor levels was determined as the relative receptor phosphorylation.

### 4.4 Results

#### 4.4.1 DMAQ as A fluorescent reporter of ATP binding-competent receptor kinases

**4.4.1.1 Optical Spectroscopy**

To gain an understanding of how DMAQ might behave in solution and the bound states, UV–vis and fluorescence spectroscopy were carried out in PBS, octanol and PEG (Figure 4.3A). Bound ligands may experience a more rigid and potentially less polar environment that can reasonably be approximated for solution spectroscopy by polyethylene glycol (PEG) and octanol. The Figure 4.3. (A) UV–vis and emission spectra of DMAQ in PEG and PBS. Emission is enhanced 23-fold in PEG relative to PBS. The lowest energy electronic transition correlates well with the predicted energy (DF B3LYP 6–31G). (B) The frontier orbital distribution and energies of DMAQ compare are remarkably similar to DCS, a well-studied stilbene that functions as a ‘turn-on’ fluorophore, suggesting similar photophysical behavior for DMAQ.
lowest energy optical transition of DMAQ in PEG is centered at 380 nm with $\varepsilon = 22,300 \text{ M}^{-1} \text{ cm}^{-1}$, while in octanol a slight blue shift ($\lambda_{\text{max, abs}} = 374 \text{ nm}$) and hyperchromic shift ($\varepsilon = 24,300 \text{ M}^{-1} \text{ cm}^{-1}$) was observed. Thus, DMAQ is comparable to commonly used fluorescent probes such as DAPI or coumarin derivatives and may take advantage of existing filter sets or laser lines for imaging purposes; at 405 nm, the excitation of commonly available violet lasers, $\varepsilon \approx 15,000 \text{ M}^{-1} \text{ cm}^{-1}$. In PBS, a marked hypsochromic shift is observed with $\lambda_{\text{max, abs}} = 324 \text{ nm}$, which is indicative of H-aggregate formation.\textsuperscript{124} The emission intensity in PEG is dramatically enhanced relative to PBS with quantum yields of photoemission ($\phi_{\text{em}}$) of 0.39 and 0.017, respectively; an identical enhancement was observed in octanol. This represents a 23-fold increase in emission intensity upon going from aqueous solution to a more viscous environment that limits the nonradiative relaxation of DMAQ. This is comparable to ON–OFF ratios observed for molecular beacon constructs.\textsuperscript{125, 126} This emission enhancement may be the result of two simultaneous phenomena. First, the absence of ground state H-aggregates reduces the likelihood of intermolecular quenching. Additionally, stilbene-like dyes possessing both electron rich and electron deficient substituents are highly sensitive to solvent polarity and viscosity due to the formation of intramolecular charge transfer (ICT) excited states.\textsuperscript{127} In the case of DMAQ, the dimethylamino group serves as the electron donor while the quinazoline moiety functions as the acceptor due to the presence of two electron-withdrawing, sp$^2$-hybridized nitrogen atoms. An additional mode of emission enhancement was anticipated if probe DMAQ docked in the catalytic site of ERBB2; the confined space of the binding pocket could serve to reduce the likelihood of a twisted intramolecular charge transfer (TICT) excited state, leading to an enhancement of
emission. Similar effects have been observed for photoexcited stilbenes such as DCS confined to the binding site of antibodies. Inspection of the frontier molecular orbitals (FMO) of DMAQ and DCS reveals that these two molecules share a similar FMO topology (Figure 4.3B) with nearly identical energies for the HOMO→LUMO transition suggesting that DMAQ would exhibit similar photophysical behavior to DCS.

In order to evaluate whether the solvent dependent increase in quantum yield is also reflected in increases in fluorescence in receptor binding, we incubated DMAQ with full length, His-tagged ERBB2 receptors which were extracted under mild lysis conditions and subsequently enriched on and eluted from Ni-NTA beads in PBS/EDTA. Differential fluorescence 3D scans of samples identified 375 nm excitation and 450 nm emission as the conditions for the best differential signal after addition of DMAQ. Under these physiological salt conditions, DMAQ gives minimal fluorescence in the absence of affinity purified receptors but exhibits a peak emission that is increased 23-fold over the free probe in PBS. Characterization of the emission and excitation spectra of ERBB2 bound DMAQ shows that its fluorescence characteristics resemble those of DMAQ in PEG with an excitation maximum of 375 nm for maximum emission at 450 nm (Figure 4.3A).

4.4.1.1 Inhibition studies

In order to correlate the ERBB2 dependent fluorescence of DMAQ in vitro with its ability to bind ERBB2 receptors in a cellular setting, we tested the potency of DMAQ as an inhibitor of ERBB2 autophosphorylation in live cells. ERBB2 overexpressing BT474 breast cancer cells were incubated with DMAQ at various concentrations for 12 h (Figure
4.4A+ B). The autophosphorylation of ERBB2 at Tyr-1139 (normalized for ERBB2 levels) was evaluated by Western Blotting. The $K_i$ for the inhibition of autophosphorylation (3.1 µM) correlates well with the saturation behavior of

Figure 4.4. (A) Saturating titration of DMAQ binding to ERBB2 ($\lambda_{ex} = 375$ nm, $\lambda_{em} = 450$ nm) compared with the relative inhibition of ERBB2 autophosphorylation in BT474 cells. B Representative Western blot of ERBB2 inhibition by DMAQ and Canertinib (CI) and constitutively active AKT after treatment with DMAQ or the dual PI3K/mTOR inhibitor: BEZ-235 (BEZ). (B) The impact on SRC activity was tested in vitro using biotinylated HNRNPK, a known SRC client; PP1 was used as positive control. (C) Titration of DMAQ dependent fluorescence in whole cell lysate containing a fusion protein of soluble ERBB2 kinase domain and mCherry with and without Canertinib pretreatment (CI) prior to lysis. Soluble kinase domain fusions in both samples were standardized by mCherry fluorescence.

fluorescence on enriched ERBB2 preparations ($K_d = 2.9$ µM). Since DMAQ targets the ATP binding pocket of ERBB receptors, we first evaluated the extent to which it may target ATP binding pockets in a relatively generic manner. Tyrosine and serine/threonine
kinases, while functionally relatively distinct share most common structural characteristics in terms of essential regulatory and catalytic elements,\textsuperscript{128} a fact that is reflected in the potency of well established pan-kinase inhibitors such as staurosporine which binds to many protein kinases with high affinity but low specificity.\textsuperscript{129} We tested the impact of DMAQ on the activity of PIK3/AKT/mTOR pathway in the setting of an MCF7 cell with constitutively active and ERBB independent AKT using the feedback phosphorylation of AKT as an established readout for AKT pathway activation. The specificity of the detection system was validated using the bifunctional (mTOR/PIK3) inhibitor BEZ235. At concentrations in which the autophosphorylation of cellular ERBB2 is fully inhibited by DMAQ, no inhibition of AKT signaling was observed (Figure 4.4B). To further evaluate the specificity of DMAQ we tested its ability to inhibit the more closely related SRC tyrosine kinase. SRC is located downstream of many receptor initiated pathways but reciprocally phosphorylates and modulates the activity of receptor tyrosine kinases. We therefore evaluated SRC activity in vitro using recombinant purified SRC and a recombinant biotinylated substrate, the heterogeneous ribonucleoprotein K (HNRNPK). Following incubation, HNRNPK-biotin is removed from the reaction and evaluated for its level of SRC dependent tyrosine phosphorylation. While the known SRC inhibitor PP1 inhibits the tyrosine phosphorylation of HNRNPK by SRC, DMAQ showed no inhibitory effect up to a concentration of 6 µM. System wide screens of inhibitor binding as well as structure comparisons of bound inhibitors to ERBB kinase domains have shown that binding specificity and affinity is not readily predictable based on the sequence conservation. This is also evident by the nature of off-target effects of existing kinase inhibitors.\textsuperscript{130} The above assays provide some
insight into the degree of specificity in binding by DMAQ. However, an even more exhaustive evaluation of potential individual kinase targets does not readily address a key concern that is unique to optical probes, namely the extent to which a large number of incremental and functionally inconsequential binding events to a large number of kinase folds adds up to a non-specific fluorescence signal that rivals the on-target signal. We therefore evaluated whether DMAQ would selectively bind to the truncated kinase domain of ERBB2 when expressed as a soluble mCherry fusion and presented in the context of whole cell lysate. The contribution of ERBB2 kinase domain to the fluorescence was evaluated through pretreatment and subsequent removal of Canertinib. Canertinib is considered specific for ERBB receptors, and furthermore reversibly modifies a unique, catalytic site proximal cysteine in ERBB1, ERBB2 and ERBB4.\textsuperscript{131} Thus DMAQ induced fluorescence that is suppressed by pretreatment with Canertinib prior to lysis can with a high degree of certainty be assigned to ERBB receptors. The equivalent recovery of soluble ERBB2 kinase domains from Canertinib treated and non-treated cells was confirmed using the fluorescence of mCherry. Despite the low abundance of ERBB2 kinase domains in lysate from less than 10\textsuperscript{6} cells, the characteristic fluorescence of DMAQ could be detected. In Canertinib pretreated cells, emission was suppressed by approximately 80\% (Figure 4.4C), indicating that at a minimum, 80\% of the obtained signal was derived from the ERBB2 kinase domain. The remainder may represent cumulative fluorescence from alternative targets, or incomplete covalent modification of ERBB2 kinase domains by Canertinib, given that it is labile chemically reactive group and inhibitory binding moiety are distinct entities. Alternatively, we cannot exclude an unfavorable but detectable mode of binding that involves covalently
coupled and tethered Canertinib but binding pocket localized DMAQ. The saturation behavior for the fluorescence of Canertinib treated samples would argue against fluorescence from a large number of low affinity targets.

4.4.2 Emission tuning of fluorescent kinase inhibitors: conjugation length and substituent effects

4.4.2.1 Optical Spectroscopy

To investigate their optical properties quantitatively, we obtained their UV−vis and fluorescence spectra in chloroform. The absorption maxima (Figure 4.5 and Table 4.1) correlate very well with the predicted values both in terms of transition energies as well as oscillator strength. The transition energies progress in a clear trend, as $\lambda_{\text{max,abs}}$ increases with increasing conjugation length. The presence of an auxochrome also serves to modulate the absorption wavelengths, as is evident in the lower transition energies for compounds possessing the dimethylamino (1a−3a) or nitro substituent (1e−3e); these modifications enhance CT character and lead to a longer wavelength absorption band lacking vibronic structure. Some vibronic structure is visible in the absorption spectra of compounds lacking an auxochrome or possessing the methoxy or cyano groups; however,

Figure 4.5. Absorption (solid lines) and emission (dashed lines) spectra of (A) 1a−1e, (B) 2a−2e, and (C) 3a−3e in CHCl$_3$. Emission intensities are given relative to 3d, which has the highest quantum yield (see Table 4.1).
these compounds possess some degree of CT in the excited state, as their emission spectra are largely devoid of vibronic progressions. Despite the presence of CT character, the allowability of the $S_0 \rightarrow S_1$ transition is relatively high for most compounds, as is evident from the good to moderate molar absorptivities. Increasing the conjugation length increases the extinction coefficient in a stepwise fashion: compounds 2a–2e possess molar absorptivities roughly twice the values observed for 1a–1e, whereas compounds 3a–3c have molar absorptivities approximately three times greater than those of 1a–1e. This trend, which reflects the relative magnitudes of transition dipoles, can be directly linked to the spatial overlap of the contributing molecular orbitals, in this case the HOMO and LUMO. In terms of optical compatibility, all of the synthesized probes are compatible with DAPI, Hoechst 33342, or blue fluorescent protein filter sets for epifluorescence microscopy, whereas 2a, 2e, and 3a–3e are optimally matched to the 405 nm diode laser.

The emission spectra of 1a–3e in chloroform are shown in Figure 4.5; most of the probes exhibit good to moderate quantum yields (Table 4.1). Emission intensities are enhanced in less polar solvents such as toluene and are reduced in more polar solvents such as acetonitrile, supporting the existence of excited states with significant CT character (data not shown). Although many of the probes exhibit emission on the blue end of the visible spectrum, several probes show longer-wavelength emission owing to their longer conjugation length and/or the presence of strong electron-donating or -withdrawing groups. Of the probes exhibiting strong emission, 3c has the bluest emission maximum (422 nm), whereas 3e has the reddest emission maximum (561 nm). Surprisingly, strong CT character does not necessarily equate to poor quantum yields, as seen for probes 1a and
2a. Indeed, 1a is the only member of the 4-phenylquinazoline series (1a−1e) of compounds that exhibits an appreciable degree of fluorescence (ϕ<sub>em</sub> = 0.37). In the 4-
styrylquinazoline (2a−2e) series, compounds possessing electron-donating groups (2a and 2b) also exhibit the highest quantum yields. With longer conjugation lengths, quantum yields of fluorescence are markedly higher and the identity of the auxochrome influences ϕ<sub>em</sub> to a lesser degree.

The overall brightness (ε·ϕ<sub>em</sub>) is one important parameter when considering the utility of the probes as optical reporters. An additional parameter, the turn-on ratio, should also be considered as a measure of the probes’ responsiveness to changes in their chemical microenvironment. Water (E<sub>T</sub>(30) = 63.1 kcal/mol, η = 0.89 mPa·s) and octanol (E<sub>T</sub>(30) = 48.3 kcal/mol, η = 7.24 mPa·s) represent two distinct environments that can be used to assess the physical properties and distribution of organic molecules. The ratios of emission intensities obtained in octanol and water are shown in Figure 4.6. Compounds 1a, 2a, and 3e showed the largest on/off ratios with enhancements greater than 50-fold; moderate ratios, between 20 and 40 were observed for 2d, 3a, 3b, and 3d. Moderate

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Table 4.1. Photophysical data for 1a-3e.
enhancements were found for the remaining members of the 2 and 3 series, whereas the
remaining 6-arylquinazolines (series 1) showed essentially no emission enhancements.

![Graph](image)

Figure 4.6. Emission intensities obtained in octanol and water reveal that several
probes are highly responsive to changes in their chemical microenvironment and
possess high ON/OFF ratios.

### 4.4.2.3 Inhibition studies

The extension of the aromatic system via the 6-position of the quinazoline core was not
anticipated to affect the key binding contacts between the probes and the ATP-binding
fold of the ERBB family. The crystallographically determined binding modes of
gefitinib,
Erlotinib,
and Lapatinib show that the 6-position is amenable to substitution
and, in some cases, decreases $k_{\text{off}}$ of inhibitors. To test this assumption, we evaluated
selected probes as inhibitors of ERBB2 phosphorylation in MCF7 cells initially using two
probe concentrations, 10 μM and 100 nM (Figure 4.7). MCF7 cells are a well-established
model system for the ligand-induced activation of ERBB2–ERBB3 heterocomplexes by
ligands of the neuregulin family (NRGβ1 in this case). Compounds 1a, 2a, 2d, 3d, and 3e
were selected because they showed the highest turn-on ratios of their respective series.
All five probes showed inhibition of NRGβ1-induced phosphorylation of ERBB2 at 10 μM; however, little to no inhibitory action was observed at 100 nM. These results demonstrate two key features of these fluorescent quinazoline probes: First, despite chemical modification, the probes remain membrane permeable and are able to access the intracellular kinase binding domain of ERBB2. Second, the pharmacophore remains an inhibitor of ERBB2 phosphorylation despite the presence of bulky extensions of the aromatic system.

We next obtained the inhibition curves of 1a, which possesses the smallest modification at the 6-position, and 3d, which possesses one of the longest arms. The $K_i$ for these probes was similar with values of 10 μM for 1a and 9 μM for 3d. A direct comparison with actual binding constants for a type I inhibitor such as Gefitinib in a cellular setting are difficult to obtain. Recombinant EGFR kinase domains have yielded an in vitro $K_d$ of approximately 1 nM. Equilibrium models in live cells treated with 10 μM Gefitinib
have yielded $K_d$ estimates of 2 to 3 nM after competition with cellular ATP was taken into account.\textsuperscript{136} By this standard, the derivatives used in our study show comparable potency. Although 1a exhibited a typical inhibition curve profile, with complete inhibition of ERBB2 phosphorylation at approximately 20 μM, higher concentrations of 3d do not lead to complete inhibition. While 3d is a larger molecule, hydrophobicity does not appear to play a role, as 1a and 3d have similar octanol/water partition coefficients, with log $P_{octanol/water} = 1.50$ and 1.55, respectively. The lack of complete inhibition may reflect alternative binding modes for the inhibitors, as similar partial inhibition resulting from competing cellular ATP has been described for Gefitinib when compared to the complete inhibition by the type II inhibitor, Lapatinib.\textsuperscript{136} We are presently evaluating the binding kinetics of these probes and will report their binding modes separately.

4.4.2.3 Binding-induced emission studies

Emission spectroscopy identified several probes (1a, 2a, and 3e) that exhibit large turn-on ratios, and the phosphorylation inhibition studies demonstrate that the modified N-phenyl quinazolines are capable of accessing the ATP-binding pocket of ERBB2. To determine if turn-on emission is observed upon binding to ERBB2, we obtained the fluorescence spectra of 1a, 2a, and 3e in PBS in the presence and absence of the soluble ERBB2 kinase domain. In PBS alone, the emission of the probes is largely quenched, whereas the addition of the ERBB2 kinase domain produced substantial emission enhancements for both of the dimethylamino-substituted probes. In the case of 1a, the emission enhancement was 4-fold when comparing all emission wavelengths, with a maximum enhancement of 10 at 435 nm (Figure 4.8). For 2a, the emission was increased by a factor
of 8 when comparing all emission wavelengths, with a maximum enhancement of 12 at 560 nm. No emission enhancement was observed for 3e despite the fact that it also exhibits solvent sensitivity and was shown to inhibit ATP binding to ERBB2. The lack of binding-induced emission enhancement may be a result of the longer conjugated arm projecting beyond the binding pocket and being exposed to the polar solvent environment.

4.5 Discussion

In summary, we have successfully demonstrated the fusion of an optical reporting element with an ERBB-targeted ATP mimic. DMAQ possesses several attractive properties as a novel biochemical tool for investigating dynamics of receptor tyrosine kinases. First, the large increase in fluorescence upon binding facilitates measurements without the need of removal of free probe. Second, DMAQ is cell permeable, which makes it a suitable starting point for the design of state specific probes for both in vivo and in vitro usage. Additionally, the quinazoline core is readily modified through either the optically active styryl arm or the

Figure 4.8. (A) 1a and (B) 2a exhibit turn-on emission upon binding to soluble ERBB2 kinase domain. In PBS alone, emission (in black) is largely quenched, whereas in the presence of ERBB2 kinase, emission (in green) is significantly enhanced; conditions: [1a] = [2a] = 1 μM; [ERBB2] = 100 nM, λex = 370 nm.
substitution at the pharmacologically relevant 4-amino position. This versatility should enable development of spectroscopically unique probes with tunable affinities or specificities for related receptor tyrosine kinases. Although designed on the structural framework of ERBB specific kinase inhibitors, the degree of binding specificity of DMAQ requires further analysis. Additional targets may be hit at higher concentrations and indeed “off target” effects have in many cases emerged as some of the primary discriminating factors in clinical efficacy. Furthermore, large numbers of distantly related molecular ATP binding scaffold could bind collectively with low affinity contributing to a significant response. However, screens against an enriched target, AKT, and against whole cell lysate containing multiple ATP binding sites suggests that DMAQ retains the selectivity seen for related 4-aminoquinazoline inhibitors. While initial tests indicate that the cumulative signal from off target effects may be low compared to the primary, ERBB-derived signal, this ratio will determine the threshold for the number of receptor molecules that can be studied. The concept of an optically responsive pharmacophore may be readily extended to other classes of biomolecules and ligands providing a complementary spectroscopic tool and bioanalytical technique for investigating biomacromolecular structure and dynamics.

We have synthesized a family fluorescent of quinazoline probes targeting the ATP-binding pocket of ERBB2 and evaluated the influence of extended conjugation as well as substituent effects through the 6-position of the quinazoline core. Our results demonstrate that the optical band gap can be manipulated by varying the conjugation length and auxochrome substitution. Depending on the auxochrome identity, the quinazoline core can function either as an electron acceptor or an electron donor to achieve polar CT
excited states. The strongest electron-donating and -withdrawing groups (e.g., dimethylamine, cyano, and nitro) yield high on/off ratios, suggesting that they are the best candidates for designing future turn-on probes. Importantly, the presence of a fluorophore arm at the 6-position of the quinazoline does not significantly attenuate the ability of the probes to function as inhibitors of ERBB2. Two probes, 1a and 2a, were successfully demonstrated as turn-on probes that can report binding to the ERBB2 kinase domain in solution. One limitation of the current family of fluorescent inhibitors is their relatively low solubility in aqueous solutions, although this is not unique to their modified structure, as Lapatinib shows significant aggregation at physiologically relevant concentrations.137
Chapter 5 ERBB2 overexpression establishes ERBB3 dependent hypersensitivity of breast cancer cells to Withaferin A

5.1 Summary

The ERBB3 receptor contributes to resistance in treatments that target ERBB2 receptor tyrosine kinase, and its levels represent an overall risk factor for unfavorable disease outcomes in breast cancer. In the absence of classic catalytic activity, it is not a target of pan ERBB kinase inhibitors. We evaluated the steroidal lactone Withaferin A (WA) for its ability to inhibit ERBB2 overexpressing cancer cell lines and complement the efficacy of target therapeutics at reduced toxicity. WA has established broad anti-cancer properties through several modes of action and has been shown effective against triple negative breast cancers. We found that ERBB2 overexpression does render cells hypersensitive to WA. While ERBB2 downregulation is one aspect of WA treatment, it is not causal for the elevated sensitivity instead WA also targets ERBB3 and therefore a crucial and highly synergistic activity in ERBB2 overexpression. ERBB3 receptor levels and its constitutive activation as well as its associated activation of AKT are downregulated by WA treatment. The simultaneous targeting of ERBB2 and ERBB3 renders cells vulnerable, even in BT474, where direct ERBB2 targeting with Lapatinib is less effective. We also demonstrated that WA interacts with CDC37 and disrupts the Hsp90/CDC37 complex with ERBB2, which is potentially the underlying mechanism of ERBB targeting by WA. In conclusion, WA or derivatives may therefore present a low toxicity addition to supplement with ERBB2 targeted therapeutics, especially in cases where ERBB3 is driving resistance or reduced overall sensitivity.
5.2 Background remarks

Overexpression of the receptor tyrosine kinase ERBB2 is a feature of many solid tumors, but most prominently in breast cancer where approximately 1/3 of patients are estimated to have highly amplified ERBB2 levels due to gene amplification. While both therapeutic antibodies and ERBB family directed kinase inhibitors are available for treatment, in consistent response rates and disease reoccurrence remain a problem for a large percentage of patients. Apart from modes of activation that completely bypass the canonical ERBB signaling pathways, a frequent route to resistance is the increased involvement of the most potent heterodimerization partner of ERBB2, the kinase impaired ERBB3 receptor. Much of the cancer supporting phenotype of deregulated ERBB3 activation relates to its exceptionally potent activation of the PI3K/AKT pathway. Resistance can take the form of increases in the ERBB3 activation by residual ERBB2 activity or receptors of other families. Complete and sustained inhibition of ERBB2 requires doses of inhibitors associated with significant toxicity concerns. Some contributions of ERBB3 signaling are apparently independent of ERBB2 and ERBB3 is now recognized as a risk factor in triple negative breast cancers. However, the strongest and best established contribution by activated ERBB3 is directly linked to its interaction with overexpressed ERBB2. Regardless of whether high efficacy is achieved by more effective targeting of ERBB2, or combination with ERBB3 directed drugs, increased potency through the use of low toxicity compounds is desirable. Although often limited in their utility as stand-alone therapeutics, natural compounds with known medicinal utility can meet this requirement. In fact the very same complex mode of action that is
often associated with their limitations as classic therapeutics may prove beneficial for their use as a sensitizer or supplementary treatment.

Withaferin A (WA) is a steroidal lactone isolated from winter cherry (Withania somnifera). The purified compound was first shown to suppress Ehrlich ascites carcinoma in more than 50% of WA treated mice. In addition, disease free survival increased when administered post treatment. Since then, WA has been studied extensively as prototype withanolide for anticancer agent treatment in various cancer models and has shown to inhibit the growth of PC-3 prostate xenografts, MDA-MB-231 xenograft models for triple negative breast cancer, PanC1 models for pancreatic cancer, and others. WA also inhibits human umbilical vein endothelia cell (HUVEC) proliferation and exerts potent antiangiogenic activity in FGF-2 matrigel mouse models of angiogenesis. This antiangiogenic property occurs at an IC50 of 12 nM, well below the concentrations required for the broader response observed across various model systems. This antiangiogenic property is linked to the covalent and degradation enhancing modification of vimentin by WA. Another known molecular target of Withaferin A is Hsp90 through covalent interaction. However, with few exceptions (such as ascites), WA treatment does not increase survival in xenograft models, and tumor suppression requires continued administration. The complex mode of action of the WA base compound is a limiting factor in the utilization as a stand-alone treatment. On the other hand, the low toxicity and broad spectrum response across cancers suggests that WA may be well suited as an amplifier or supplementary treatment alongside targeted drugs.
For breast cancer cells, a response at higher WA concentrations had already been observed for the MDA-MB-231 triple negative (estrogen receptors -, progesterone receptors -, and ERBB2 -) model system. However, the example set forth by the targeting of angiogenic responses by WA also showed that much higher levels of sensitivity can be found in a cell type specific setting. Within breast cancers, ERBB2 overexpression defines a clearly discernable group, both in terms of their expression profile and tumor phenotype. We therefore evaluated whether ERBB2 amplified cancer lines share the sensitivity to WA displayed by MDA-MB-231 or exhibit a distinct WA sensitivity. This analysis demonstrated that ERBB2 overexpression confers an elevated level of WA sensitivity in a manner that is correlated to the cells level of dependency on synergistic signaling by ERBB3 and ERBB2, even when the sensitivity to Lapatinib is reduced.

5.3 Materials and methods

**Cell lines.** The effects of WA was tested on several natural occurring breast cancer cell lines carrying different ERBB2 and ERBB3 levels, including MCF7, SKBr3, BT474 and MDA-MB-231. MCF7-B2 cells expressing moderately elevated ERBB2 were generated by retrovirally infecting MCF7 with human ERBB2 cDNA. All cell lines were maintained in 1X RPMI -1640 medium supplemented with 10% fetal bovine serum, 2 mM/L L-glutamine and 0.05 mg/ml gentamicin.

**Reagents.** WA was purchased from Chromadex Inc. and dissolved in 100% ethanol for experiments. Lapatinib was purchased from LC Laboratories and dissolved in 100% DMSO.
The ERBB3 GFP construct was generated by cloning ERBB3 cDNA into a pFlag-myc-CMV-19 vector. GFP sequence was attached at the C-terminal tail of ERBB3. The mCherry construct was purchased directly from Clontech.

Western blot antibodies against Erk 1/2 MAPK (9102), AMPK (2532), GAPDH (8884), phospho-ERBB3 (4791) were purchased from Cell Signaling. ERBB4 (3412) and phosphor-P38 (1229) were products of Epitomics. ERBB2 (ab8054) and phosphor-ERBB2 (ab53290) antibodies were obtained from Abcam. ERBB3 antibody (sc-285) was purchased from Santa Cruz Biotechnology.

**Cell proliferation and viability.** Various methods were used to detect cell proliferation or viability in response to WA treatment. In a subset of experiments, MTT was used as an indicator of cell viability. Tumor cells were seeded in 96-well plates at a density of 5 x 10^4 cells per well. After overnight adherence, the cells were treated with increasing concentrations of WA as indicated. After 48hr incubation, the cells were washed in PBS and new phenol red negative media was added containing 20 μl of MTT (5mg/ml stock). Cells were incubated at 37 °C for 1.5 hr. The media was removed and DMSO (150 μl) was added to solubilize the formazan salt formed. Quantification of formazan salt formed was determined by measuring OD at 560 nM and 690 nM reference using a microplate reader. Background control consisted of media containing MTT without cells.

Flow cytometric analysis of dead cells was done using 7-AAD nucleic acid stain (eBioscience). 7-AAD is excluded from viable cells but stains DNA of dead cells with compromised membranes. Cells were treated with 10 μM WA at indicated time points or treated with vehicle (100% ethanol) or WA. Following treatment, both adherent and non-
adherent cells were collected, washed with PBS twice and stained with 5 μl 7-AAD in 100μl volume and immediately analyzed on the LSRII flow cytometer.

Alamar Blue Assay was used in drug combination assays with WA and Lapatinib. BT474 and SKBr3 were seeded in 96-well plates at a starting quantity of 3 x 10³ and 6 x 10³ per well respectively with the presence of WA and Lapatinib of various concentration indicated. Alamar Blue reagent of 10% culture volume was added to the cells 48 hours after inoculation. After 4 hour incubation, absorbance at 570 nm was measured by using a microplate reader. Spare wells on each plate without cell inoculation but receiving medium and Alamar Blue were used as controls for background subtraction.

**Flow cytometric analysis of cell surface ERBB2.** Cells were plated overnight at a density of 5 x 10⁵ in six well plates and treated next day with 10μM WA at indicated time points followed by Fc receptor blocking using CD16/32 antibody (eBioscience) for 20 mins on ice to prevent non-specific antibody binding. Cells were then washed and incubated with anti-neu (ER23) antibody directed towards the extracellular domain of human ERBB2 (Santa Cruz Biotechnology) for 30 minutes on ice at a 1:50 antibody dilution. Cells were subsequently washed and incubated with goat anti mouse Alexa Fluor 488 secondary antibody (Molecular Probes) for 30 mins on ice at a 1:200 antibody dilution. Finally cells were washed and fixed in a final concentration of 1% p-formaldehyde. Cells were assessed on LSRII flow cytometer and analyzed using Flowjo software (Tree Star Inc.). Analysis was performed on 10,000 events per treatment sample.

**Western blot.** Cells were seeded in six well plates with starting quantity of 3 x 10⁵. The next day, cells were treated with 10μM WA or vehicle. After 4 hours of drug treatment,
cell lysates were collected by either using mild lysis buffer (20 mM Tris, 137 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10% glycerol) or SDS sample buffer. Immunoblots were performed using antibodies specific against proteins listed in result sections. For comparison across cell lines, equal amount of total lysate protein (estimated by BCA assay) were loaded.

**Transfection of SKBr3+ERBB3 and flow cytometry viability analysis.** SKBr3 cells were seeded in six well plates one-day prior with starting quantity of 2x10^5. Transfection of ERBB3-GFP and mcherry constructs was done by lipofectamine 2000 (11668019, Life Technologies). Cells were treated with 1 μM of WA on the same day of transfection and 24, 36, 42, 48 hours afterwards. Following incubation cells were stained for ERBB2 expression using monoclonal anti-ERBB2 (ER23) antibody (Santa Cruz Biotechnology) directed towards the extracellular domain of ERBB2 (1:50) followed by Alexa Fluor 647 secondary antibody (Molecular Probes 1:200). Fixable viability dye eFluor 450 was used to distinguish live vs. dead cells according to manufacturer’s instructions (eBioscience). Cells were fixed using a final concentration of 1% p-formaldehyde and assessed on the LSR II flow cytometer. Analysis was done on 50,000 events using Flowjo software (Treestar Inc.).

**Statistics.** Graph Pad Prism software was used to perform all statistical analyses. At least two independent experiments were performed and mean ± SD was calculated. Statistical differences between control and experimental groups were determined by an unpaired Student’s t test. Significance is denoted as *, p<.05 **, p<.01 ***, p<.001 in some experiments. The IC50 value for cytotoxicity was accessed by non-linear curve regression analysis.
5.4 Results

5.4.1 ERBB2 overexpressing breast cancer lines exhibit elevated sensitivity to WA

With WA sensitivity already reported for the triple negative line MDA-MB-231, we compared a set of breast cancer lines representing both ERBB2 amplified and non-amplified cancers for the impact of WA on proliferation. These lines included ERBB2 gene amplified and overexpressing SKBr3 and BT474, non ERBB2 overexpressing MCF7, and triple negative MDA-MB-231. With regard to ERBB2/ER status, SKBr3 represents an extreme scenario of an ER negative and highly ERBB2 overexpressing line. BT474 is frequently used as a model for ERBB2 overexpression and drug resistance evaluation but features lower levels of ERBB2 than SKBr3. The ER (Estrogen receptor) status of BT474 is inconsistent, being reported as either negative or marginally positive.

Figure 5.1. Growth of several breast cancer cell lines are inhibited differentially by 48 hour WA treatment, based on an MTT based proliferation assay, presented as both bar (A-E) and curve (F) diagram. The ERBB2 overexpressing lines SKBr3 and BT474 display higher sensitivity. MCF7-B2 stably expresses additional ERBB2 at lower levels and displays intermediate sensitivity to WA compared to MF7 parental line and the triple negative line MDA-MB-231.
The ER positive line MCF7 was chosen because it expresses very low and ligand responsive levels of ERBB2 and its primary hetero dimerization partner, ERBB3. In addition, ERBB2 transfected MCF7 cells are widespread isogenic model system for the impact of ERBB2 amplification. MDA-MB-231 was used as a triple negative line.

Within this set of cell lines, pronounced differences were apparent in the sensitivity when WA was added at concentrations up to the micromolar concentration used in prior studies (Figure 5.1). For MCF7 cells, initial inhibition of proliferation leveled out at approximately 50% of control. Over this concentration range, MDA-MB-231 exhibited a comparable sensitivity in the micromolar range, consistent with previous reports. A much more pronounced response occurred in the two ERBB2 overexpressing lines, SKBr3 and BT474. MCF7-B2 represents an engineered MCF7 derivative with moderate but constitutive overexpression of ERBB2. Its sensitivity was intermediate to ERBB2 gen amplified lines and MCF7 control in that it showed a later onset of inhibition but higher final sensitivity than MCF7.

5.4.2 WA induces downregulation of ERBB2

ERBB2 is a major driver of enhanced proliferation under conditions of stable overexpression and both BT474 and SKBr3 show enhanced sensitivity. We therefore evaluated whether WA acts by reducing the levels of ERBB2. In a direct comparison of MCF7 cells with their ERBB2 overexpressing derivative line MCF7-B2, ERBB2 is indeed downregulated rapidly with 4 hours of treatment with WA and this downregulation increased only modestly after prolonged incubation (8h). The WA induced removal of cell surface ERBB2 is more pronounced for MCF7-B2 and leaves
both cell lines with a matching comparable receptor levels in both FACS (Figure 5.2A+B) and western blots (Figure 5.2C). Both SKBr3 and BT474 show WA induced downregulation with difference in the rate and degree of downregulation (Figure 5.2D+E).

For BT474 the apparent lower response by FACS may reflect a higher and WA independent rate of internalization, triggered by EDTA (Versine) induced detachment and processing prior to FACS analysis. Overall, WA does induce downregulation of ERBB2 which is likely to contribute to its effects, but this alone does not account for the differences seen in the sensitivity of cells to WA.

Figure 5.2. WA rapidly lowers ERBB2 levels in overexpressing cells. Cell surface localized ERBB2 levels in MCF7 parental and ERBB2 transfected MCF7 after 4 hours (A) and 8 hours (B) of WA treatment with 10 µM WA were measured by flow cytometry. ERBB2 levels in MCF7 parental cells were reduced by 35.2% and 35.1% following 4 hour and 8 hour WA treatment. More pronounced reduction in ERBB2 levels was found in MCF7-B2 cells. (C) The loss of cell surface ERBB2 is also reflected in total cell lysate. A similar but quantitatively different downregulation of cell surface ERBB2 occurs in SKBr3 (D) and BT474 cells (E).
5.4.3 WA induces downregulation of receptor levels and phosphorylation of both ERBB2 and ERBB3

While ERBB2 is a strong contributor to cell proliferation, its oncogenic potency is very dependent on the available complement of hetero dimerizing ERBB family members. Especially the kinase impaired ERBB3 receptor does not only convey ligand sensitivity and allosteric activation, it complements the MAPK centered signal transduction of ERBB2 through its exceptionally strong signal into the PI3K/AKT pathway. While never

![Figure 5.3](image)

Figure 5.3. WA reduces ERBB2 and ERBB3 levels in all the cell lines tested and completely abrogates constitutively phosphorylated ERBB2 and ERBB3 in ERBB2 overexpressing cell lines (SKBr3 and BT474) (A). In MDA-MB-231 cell line, WA increases the phosphorylated ERBB2 in spite of the abolished ERBB2 (B). WA treatment produces a ligand stimulation like pattern of MAPK activation in BT474 with maximal activation at 60 minutes post WA addition (C). Although starting with differential constitutive p-Erk1/2 level, WA induced elevation of p-Erk1/2 is conserved across SKBr3, BT474 and MCF7 cells (A). WA also abolishes AKT levels in SKBr3, BT474 and MCF7 cells, which translates to completely removed p-AKT in the two ERBB2 overexpressing cell lines (A). ERBB4, AMPK, Erk1/2 and GAPDH levels are not affected by WA treatment implying target specificity of WA (D).
overexpressed in isolation, ERBB3 has emerged as a key player in resistance against ERBB2 or EGFR directed treatment. Figure 5.3 shows the response for both ERBB2 and ERBB3 in terms of receptor levels and phosphorylation state. For ERBB2 receptor levels, figure 5.3A shows two different exposures to convey the large range of receptor expression from SKBr3 to MCF7, covering more than two orders of magnitude when normalized for protein content. WA treatment triggers a comparable relative downregulation of ERBB2 across all three cell lines. For SKBr3 and BT474, the ERBB3 is completely removed while MCF7 shows a strong but incomplete removal. For the constitutively phosphorylated species of both ERBB2 and ERBB3 the decrease is even more pronounced. The loss of phosphorylated ERBB2 is however not reflected in the steady state levels of phosphorylated MAPK. Here, phosphorylation is elevated from very different starting levels in all three cases. The increase in pMAPK levels despite a loss in levels of both total and phosphorylated receptors, matches reports of WA activation of MAPK in triple negative lines.\textsuperscript{145} For MDA-MB-231 as similar increase occurs, albeit at a smaller scale due to high preexisting levels of pMAPK (Figure 5.3B). Steady state levels of pMAPK are necessarily a good reflection of the level of MAPK mediated signaling. Upon ligand stimulation proliferation enhancing MAPK activation typically shows a very pronounced temporal response pattern with a large spike in activation as early as 15-30 minutes only to fall back to almost starting levels upon continuous stimulation, despite being critical for a proliferation response. Low levels of pMAPK may therefore also reflect a rapid rate of pMAPK cycling. To investigate whether such WA may alter pMAPK levels in a similar manner, we measured the temporal response of BT474 to WA (Figure 5.3C). BT474 have an exceptionally low
level of steady state pMAPK, relative their high level of ERBB2 activation. Upon addition of WA, we observed a ligand like response with a pronounced increase at one hour and subsequent drop in pMAPK. A qualitative difference in the WA response occurs for AKT. While AKT levels decrease in all three lines, this response is associated with a decrease in pAKT levels only for SKBr3 and BT474. Along with its partial removal of ERBB3, very low starting levels of constitutively activated AKT increase in MCF7. The response of ERBB2, and ERBB3 and AKT to WA is not system wide. Levels of ERK1/2 and AMPK are not altered while the homologous ERBB4 receptor is far less sensitive than ERBB3, measured under conditions of partial downregulation (Figure 5.3D).

5.4.4 WA complements Lapatinib with targeting Lapatinib inaccessible ERBB3 in cells

Given that both ERBB3 and pERBB3 are rapidly decreased through WA addition, the rapid response of SKBr3 cells may reflect the reliance on a much smaller pool of ERBB3 while the increased long
term sensitivity of BT474 reflects the higher degree of dependency. Targeting the
codependency or ERBB2 and ERBB3 could provide a complementary mode of inhibition
to catalytic side inhibitors against ERBB2. To test this hypothesis, we evaluated the
combined effects of WA and Lapatinib (Figure 5.4). Lapatinib is a non-covalent, inactive
state stabilizing kinase inhibitor targeting ERBB2, EGFR and to a lesser extent ERBB4
but EGFR and ERBB4 are present in SKBr3 cells at very low levels. WA was kept
constant at 0.5 or 1.5 µM. Consistent with earlier data (Figure 5.1), the initial inhibition
of BT474 by 0.5 µM WA alone was approximately 20% higher than that of SKBr3. With
1.5 µM WA, inhibition was comparable for both cell lines. Compared to BT474,
increasing concentrations of Lapatinib alone had a large impact on SSKBr3 in which
constitutive ERBB2 signaling is only minimally supported by ERBB3. At low
concentrations of Lapatinib the combined inhibitory action of both drugs is not additive.
Instead WA mediated inhibition appears dominant. Additional inhibitory contributions by
Lapatinib become only apparent at concentrations above 1 µM. These observations would
suggest that prolonged exposure to lower concentrations of WA (compared to short term
treatment with 10 µM in Figure 5.3) ERBB3 is the primary and Lapatinib inaccessible
target. In the absence of WA, the partial inhibition of ERBB2 by lower concentrations of
Lapatinib is in part mitigated by the continuing signal of Lapatinib insensitive ERBB3
receptors. This ERBB3 mediated enhanced rug resistance is eliminated by WA. The time
delayed but ultimately higher sensitivity of BT474 cells at 0.5 µM WA and Lapatinib
concentrations above 1 µM may therefore reflect the ERBB3 status which provides
additionally lower sensitivity to Lapatinib alone and higher sensitivity to the combination
treatment.
5.4.5 Additional ERBB3 establishes hypersensitivity of SKBr3 to WA

Our initial evaluation of cell proliferation in the presence of WA occurred after 48 hours, but studies on the acute response of receptor levels included much earlier time points at which differences surfaced between SKBr3 and BT474. We therefore evaluated the impact of WA on dye exclusion as a function of time at a fixed concentration of WA (10 µM). At this concentration, both cell lines exhibit comparable endpoints in a 48 hour growth assay and rapid changes in protein levels (Figure 5.3). Figure 5.5A shows the percentage of cells that exclude 7AAD as a function of time. When exposed to WA, SKBr3 cells respond remarkably fast to inhibitor treatment. The time scale at which the intial uptake occurs for SKBr3 indicates changes in channel mediated uptake (ref) rather than a compromise of membrane integrity with both events overlapping at later time points. Regardless of the mechanism of rapid dye uptake in SKBr3, the WA induced response shows a pronounced difference to BT474 which show a higher sensitivity in longer term growth assays but display a very pronounced time delay in their dye exclusion response (Figure 5.5A). SKBr3 and Bt474 represent ER negative and ERBB2 positive and luminal B (ERBB2 and ER positive) lines respectively. However, besides their more complex system wide differences, both cell lines differ also on the extent that they utilize ERBB3 as an enhancer of constitutive ERBB2 signaling (Figure 5.3A).

Since both cell lines represent different breast cancer subtypes and differ in many aspects beyond ERBB2 and ERBB3 signaling, a direct comparison is difficult. We therefore tested the impact of WA in an isogenic SKBr3 model carrying additional recombinant ERBB3 (Figure 5.5B-F). Since contributions by EGFR or ERBB4 in SKBr3 are minimal, certainly relative to the massive overexpression of ERBB2, this approach has been used
to establish a model system for ERBB3 mediated resistance to ERBB2 targeted therapy. Receptor levels and viability were determined at a constant concentration of 1 µM WA using FACS analysis. This concentration emphasizes the ERBB2 overexpression associated response to WA. The nature of the gating ensures that all cells carry at least detectable levels of recombinant ERBB3-GFP. Additional gating on cotransfected Cherry fluorescent protein did not change the outcome but reduced the number of identified cells and statistical power (data not shown). WA was added at different times, but all cells were analyzed at the same time point post transfection, thus correcting for WA unrelated,  

Figure 5.5. (A) SKBr3 show a very rapid response to WA in a 7-AAG exclusion assay while the BT474 response is time delayed. (B) Transient transfection of SKBR3 with ERBB3 sensitizes it to WA treatment. Representative profiles of ERBB3-GFP fluorescence and ERBB2 staining of three samples receiving 0, 24 and 48 hour of 1 µM WA treatment are shown in B, C and D. (E) Relative percentages of subpopulation (normalized to 0 HR setting) are plotted in panel D indicating a significant drop of (ERBB2\(^H\)/ERBB3\(^H\)) population post 24 hours. (F) Consistently, a more pronouncing increase in the percentage of dead cells was observed in (ERBB2\(^H\)/ERBB3\(^H\)) population post 24 hours of WA treatment. (G) 1 µM WA treatment has negligible effect on the ERBB2 level in SKBr3. ERBB3 shows a reduction post 24 hours.
temporal changes of expression levels represented in the non-treated control (0 h). Total cell numbers were significantly reduced after 48 hours of WA treatment, consistent with the earlier growth assays (Figure 5.1). With comparable counting events, the FACS analysis does therefore determine differences in the rate of decrease between cell populations. Cells with the lowest but detectable ERBB3 levels (ERBB2\textsuperscript{H}/ERBB3\textsuperscript{L}) effectively represent the baseline for SKBr3 sensitivity to WA. In contrast to the receptor levels determined by FACS at 10 µM, no shift towards lower cell surface receptor levels was observed at early time points (Figure 5.5B+C). This reduced contribution of receptor downregulation at 1 µM WA is also evident by western blot analysis of the entire population (Figure 5.5G). At 24 hours, a depletion of the (ERBB2\textsuperscript{H}/ERBB3\textsuperscript{H}) population is evident and at 48 hours this population has all but disappeared compared to the non WA treated control (0 h) (Figure 5.5D). With (ERBB2\textsuperscript{H}/ERBB3\textsuperscript{L}) cells now representing almost the entire population, levels of ERBB3 receptor in total cell lysate have also declined accordingly (Figure 5.5G).

The accelerated loss of the (ERBB2\textsuperscript{H}/ERBB3\textsuperscript{H}) population (Figure 5.5E) may represent an actual loss of viable or dividing cells in this population. Alternatively it could represent a more rapid decrease in ERBB3 receptor levels, thereby converting (ERBB2\textsuperscript{H}/ERBB3\textsuperscript{H}) to (ERBB2\textsuperscript{H}/ERBB3\textsuperscript{L}) cells. To address this question, we combined receptor based sorting with a measurement of the percentage of dead cells, as determined by 7AAD exclusion (Figure 5.5F). This analysis indicates a disproportionate increase of dead cells with the (ERBB2\textsuperscript{H}/ERBB3\textsuperscript{H}) population. Combined these data suggest that the decrease in the (ERBB2\textsuperscript{H}/ERBB3\textsuperscript{H}) population does indeed indicate a higher sensitivity to WA, and not merely drug induced receptor downregulation. Furthermore, it
demonstrates that against a backdrop of ERBB2 overexpression, higher levels of ERBB3 convey enhanced, but time delayed sensitivity to WA, a response pattern comparable to BT474.

5.4.6 WA physically interacts with CDC37 and disrupts Hsp90-CDC37 association from ERBB2

Both ERBB3 and ERBB2 have been shown to be client of CDC37 and require CDC37, Hsp90 association at their nascent state or nascent and mature state.⁵⁴, ⁵⁵ At the same time, Hsp90-CDC37 complex is reported to be one of WA’s potential molecular targets.⁶⁸, ¹⁴⁴ To test the possibility that WA downregulates ERBB2 and ERBB3 via targeting the chaperone complex, we initiated an coimmunoprecipitation of ERBB2 with the presence of WA pretreatment (Figure 5.6A). WA partially disrupts both Hsp90 and CDC37 association of ERBB2 at a moderate concentration (5 µM). Higher efficiency is achieved at elevated WA concentration (10 µM). However, the disruption is never as complete as that of Geldanamycin (a Hsp90 ATP binding pocket inhibitor), indicating different action modes. Proportionally, CDC37 shows more pronounced release from ERBB2 than Hsp90,

![Figure 5.6](image)

Figure 5.6. (A) CoIP of ERBB2-bio, HSP90 and CDC37 after 30 mins Geldanamycin or WA treatment. (B) SPR saturation curve of WA binding to CDC37 (black) and unrelated controls (red/green).
implying CDC37 as the primary target of WA. Figure 5.6B shows a SPR saturation curve of WA binding to CDC37 immobilized surface. The specificity of WA interaction is demonstrated by surfaces coated with two unrelated control proteins.

5.5 Discussion

A previous study had demonstrated that the triple negative line MDA-MB-231 as well as the ERBB2 negative MCF line are sensitive to WA. We now demonstrate that this sensitivity does not only extent to ERBB2 overexpression, but ERBB2 positive lines exhibit heightened sensitivity. Furthermore, WA treatment does invoke the previously reported MAPK activation that has been associated with the sensitivity exhibited by triple negative breast cancer lines. We also observe an increase in pERK1/2 levels but this response is not proportional to the heightened WA sensitivity. Compared to ERBB-ligand induced activation of ERK1/2, four hours represent a very late time point at which steady state levels of pERK1/2 has in most cases already declined. For BT474, we indeed observed a ligand like response pattern for WA (Figure 5.3C). One of the reported actions of WA has been an increase in ROS. The steady state phosphorylation status represents the equilibrium between rapid cycles of phosphorylation and dephosphorylation, which is shifted by the inhibition of protein phosphatases by ROS. However, for the responses we observed, this is unlikely to be causal based on the ligand like time profile of WA induced activation (Figure 5.3C) as well as the pronounced qualitative differences by which different protein kinases are impacted.

Both MCF7 and MDA-MB-231 show comparable WA sensitivity at higher concentrations. Compared to both SKBr3 and BT474, both show already relatively high
steady state levels of pMAPK. This may in part explain why in our case we did not observe a pronounced increase of ERK1/2 activation in MDA-MB-231. The determination of steady state pMAPK levels does not capture differences in the rate of cycling between activated and non-activated states. This may account for the frequently observed and counter intuitive low relative levels of constitutively activated MAPK in the presence of high ERBB2 overexpression. Regardless of the differences in starting levels, all cell lines show qualitatively an increase in MAPK activation while the changes in AKT activation differ qualitatively. The ERBB2/ERBB3 signaling pair is the strongest proliferation enhancing combination among ERBB receptors. A ligand activation of ERBB2/ERBB3 stands out by very strong and “balanced” activation of both the ERBB2 driven MAPK and ERBB3 driven AKT pathway while overexpression of ERBB2 in isolation is not tumorgenic. In addition, overexpression of ERBB2 in isolation is not well tolerated in most model systems. While SKBr3 and BT474 differ in the primary target of WA driven changes, both the substantially shifted out of the preexisting balance by opposite changes to the levels of pMAPK and pAKT, in contrast, the same changes in MCDF7 cells are unidirectional and far more modest. Signaling through AKT is central to many aspects of cellular metabolism. As a means of creating a signaling imbalance large changes in AKT activation appears to convey a slower but ultimately more potent means of inhibition.

The loss of ERBB3, even at low concentrations of WA would suggest that the codependency of ERBB2 overexpressing lines on its heterodimerization partner is being targeted. It has become clear that ERBB3 is not only needed for ERBB2 to display its full oncogenic potential, but is also an important contributor to resistance to ERBB2 directed
The heightened sensitivity to WA could therefore reflect the increased dependency of ERBB2 overexpressing lines on ERBB3. This would also explain why WA responsiveness supersedes Lapatinib, especially in relatively Lapatinib insensitive lines such as BT474. The importance of ERBB3 in conveying WA sensitivity was further confirmed by the introduction of additional ERBB3 into SKBr3 cells. However, these studies also suggest that the sensitivity conveyed by elevated levels of ERBB3 may go beyond the loss of important ERBB3 contributions that the cells may have become accustomed to. The time course of the experiment does not allow for such a dependency to develop. Instead the accelerated loss of cells with high ERBB3 levels, which is accompanied by a loss of ERBB3 and an increased rate of cell death, suggest that elevated levels of ERBB3 may directly convey elevated sensitivity to WA. Whether this reflects potentially cytotoxic product of accelerated ERBB3 degradation or the cells inability to adjust to successive reversals in metabolic control by ERBB3 introduction, followed by equally rapid removal will require further investigation. Regardless of the specific mechanism of ERBB3 mediated action, WA does target ERBB2 overexpressing cancers in a concentration range that is well below those at which it has thus far exhibited valuable anti-cancer properties. In addition it provides a second line of attack in cancers where ERBB3 contributes to resistance or overall aggressiveness. Specific, non catalysis targeted therapeutics such as ERBB3 specific antibodies may provide on route to address the problem of ERBB2/ERBB3 synergy. However, these approaches are costly and it is not clear to what extent ERBB3 will be able to evade interference based on targeted binding events. WA or similar supplementary treatments could greatly enhance ERBB2 treatment with low intrinsic toxicity.
Withaferin A has been shown in the past to have general anti-tumor properties, and different molecular targets have been implicated. Combined with the generally low toxicity this makes WA a good candidate for a drug that can support or supplement other targeted therapeutic approaches. From existing studies on WA, it is apparent that the extent to which a given targeting event becomes dominant differs between cancers. Consequently, the best possible choice of targeted drugs to be combined with WA is cancer type dependent.

CDC37 and Hsp90 are required for both the maturation and stability of ERBB2. Silencing of CDC37 or inhibition of Hsp90 functions have been shown to induce extensive degradation of ERBB2 and other oncogenic kinases. Because of this, targeting this chaperone complex has been proposed to treat multiple cancer types. In this study, we demonstrated that WA, at moderate concentration (5 µM), targets CDC37 and partially disrupts the Hsp90, CDC37 association of ERBB2 after 30 mins of pretreatment. This disruption translates to receptor turnover and signaling suppression observed 4 hours post WA treatment and eventually leads to reduced cellular viability. As a kinase specific cochaperones, targeting CDC37 leads to a general effect on its clients. We indeed observed that AKT, a CDC37 client, and its activation are abrogated. These general effects caused by targeting CDC37 and other molecules establish a basal cellular inhibition that might sensitize cells for elevated inhibition through targeting cancer dependent signaling pathway, such as ERBB2/ERBB3 signaling for breast cancer.

As a drug, unmodified WA has clear limitations such as its stability in serum. However, studies that demonstrate its efficacy may spur the development of better suited derivatives. In the evaluation of these derivatives, it will be important to keep in mind
that qualitatively distinct modest of actions exist for WA in different cancer cell settings. A knowledge of the distinguishing parameters that characterize the WA response will be critical in such pursuits.
Chapter 6 Conclusion and Significance

What is the clinical implication of this work?

CDC37 has been implicated as a novel and promising target for cancer treatment in multiple circumstances. However, there has been no successful development of small molecule based inhibitors against CDC37, due to the lack of commonly targetable structural elements such as the ATP binding pocket. As a naturally occurring compound, WA simultaneously inhibits both ERBB2 and ERBB3 signaling pathways. This presents WA, or derivatives with improved pharmacokinetics, as a well suited supplement to ERBB2 targeted therapeutics, especially in cases where ERBB3 involvement is driving resistance or reduced overall drug sensitivity. However, the potential application of WA in cancer treatment is not limited to breast cancers. Our study of the CDC37-WA interaction provides a starting point for the rationale development of CDC37 specific small molecular inhibitors.

What structural feature(s) on kinases is CDC37 recognizing?

Despite the known role of CDC37 in differentiating kinases and non-kinases for the later generic chaperone complex, the molecular mechanism underlying the class specific recognition of over 300 kinase clients is not well understood. It has been hypothesized that CDC37 interacts with certain structural features that are present universally in kinases. Activation state specific recognition by CDC37 might be one of the working models. Protein kinases are composed of similar structural blocks. The catalytic domains of different kinases adopt similar structures when they are in their active state. By contrast, different kinases in their inactive state demonstrate remarkable variety in their
catalytic domains. This makes the specific recognition of the full range of activation state conformations impractical.

The small molecule kinase inhibitors Lapatinib and Canertinib freeze ERBB receptor tyrosine kinases in their inactive and active states respectively. For Braf, the V600E mutation leads to a constitutively active catalytic state. These activation state altering changes all result in enhanced CDC37 interaction (data not shown). This would suggest that structural flexibility or rigidity, not the nature of a specific defined state, is being sensed by CDC37. And that a loss in structural flexibility correlates with higher CDC37 interaction.

However, further stabilization of the active conformation of Braf$^{V600E}$ by Vemurafenib abolishes CDC37 interaction (data not shown, manuscript in preparation). This implies other components beyond conformational rigidity for the recognition by CDC37. The K723M mutation of ERBB3 is located in the ATP binding pocket and causes a tighter interaction of CDC37 with ERBB3. This suggests that the nature of the ATP binding pocket, or conformational changes related to its alteration, are recognized by CDC37. In fact all ATP pocket distorting modifications we analyzed either diminished (for Vemurafenib) or increased (for Lapatinib, Canertinib and K723M) CDC37 interactions. The differential binding by CDC37 may be determined by the extent of distortion. While tolerable structural changes elevate CDC37 association, severe perturbation diminishes CDC37 binding. The ATP binding site locates to the cleft between the N and C –lobes of kinases and sits beneath a highly conserved P-loop. In this case the structurally uncommitted N-terminus of CDC37 may serve as the structural element that is capable of accessing and sensing the ATP binding pocket of kinases.
How are the quality controls of the ER lumen and cytosol coordinated?

ERAD starts with the recognition of misfolded or defective protein substrates at the ER and ends with their degradation by proteasomes in the cytosol. These two steps are connected by the requirement to first remove proteins from the ER, a process that is usually referred as retro-translocation. The retro-translocation of transmembrane proteins such as ERBB receptor tyrosine kinases is more complicated than that of soluble proteins. In the cytosol, Hsp70/40 chaperones assist the interaction of misfolded kinases with E3 ubiquitin ligase CHIP and Doa10p (yeast). For a folded yet structural defective kinase, Hsp90 recruits Cullin5 E3 ubiquitin ligase. At the same time, the glycosylated extracellular domain of ERBB receptor tyrosine kinases undergoes folding and quality control through the calnexin/calreticulin cycle.

Defective ERAD substrates are handed over to the retro-translocon complex, which includes glycosidase and ER associated ubiquitin ligases, such as Cullin5. The ER lumen specific and cytosol specific quality controls integrate post ubiquitination at the cytosol side. The 19S cap of proteasome recognizes and is able to directly extract the poly-ubiquitinated ERAD substrate. Alternatively, the CDC48/p97 ATPase plays an active and direct role in the extraction of several ERAD substrates. The retro-translocation of soluble ER resident ERAD substrate is usually mediated by a molecular channel on ER membrane. However, integral and transmembrane substrates may be extracted in channel independent manners and involve cleavage/chop at their cytoplasmic portion.

The quality controls at both sides occur in parallel and have their distinct pools of cochaperones for substrate recognition, folding assistance, and direction to ERAD.
However, the confirmation of correct folding and modifications as well as the determination to export need to be accomplished at comparable time scale. This merger in processing could provide a means to coordinate quality controls on both sides of the ER. The failure of the ERAD complex on either side of the ER to dissociate would signal a problem in quality control. Its continued chaperon association at the time of ER-Golgi transfer could represent the trigger for degradation at this stage in quality control. Our data of the tight association of K723M with CDC37 is also in line with this model.
Reference


