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A Role for Cdc37 in EGFRvIII Biogenesis

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A Role for Cdc37 in EGFRvIII biogenesis

By

Stephen Scales

A thesis submitted to

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Abstract

The mutant epidermal growth factor receptor, EGFRvIII, is associated with tumour aggressiveness and drug resistance in glioblastoma. Our lab has shown that the molecular chaperone Hsp90 interacts with nascent EGFRvIII, and that EGFRvIII expression and function is dependent upon Hsp90 activity. In tumours, Hsp90 is found exclusively in an active form, where it is bound to cochaperones. One of these cochaperones, Cdc37, was found in the Hsp90/nascent EGFRvIII complex. Cdc37's role is to recruit certain kinase proteins to Hsp90 for folding. Its ability to bind these kinases is thought to be dependent upon phosphorylation of serine 13 in its client-binding domain by the kinase CK2. Here I have shown that Cdc37 is a limiting factor in EGFRvIII maturation, and its absence or inactivity may limit recruitment to Hsp90 and result in degradation. Using mutant constructs designed to mimic non-phosphorylated and phosphorylated Cdc37, I evaluated the role of Cdc37 phosphorylation in the stability of nascent EGFRvIII. Expression of the phospho-mimetic Cdc37 construct raised protein levels of nascent and mature EGFRvIII, while the non-phosphorylatable Cdc37 S13A mutant could not. Approximately 100 times more of the phospho-mimetic construct bound to nascent EGFRvIII than did the non-phosphorylatable form, and this increased Cdc37 binding was associated with more Hsp90 binding as well. Treatment of cells with the selective CK2 inhibitor, TBB, lowered the amount of Cdc37 and Hsp90 bound to nascent EGFRvIII, and reduced EGFRvIII protein levels. Taken together, these data indicate a positive role for Cdc37 and CK2 in EGFRvIII biogenesis, and suggest that Cdc37 phosphorylation may be a key factor in the recruitment of client to the Hsp90 complex.

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Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Cdc37 WT	Wild-type Cdc37
Cdk4	Cyclin dependent kinase 4
CK2	Casein Kinase 2
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFRvIII	EGFR mutant lacking amino acids 6-273
ER	Endoplasmic reticulum
GA	Geldanamycin
Hop	Hsp70/Hsp90-Organizing Protein
HRI	Heme-regulated eIF2-α kinase
Hsp70	Heat shock protein 70
Hsp90	Heat shock protein 90
PI3K	Phosphoinositide-3 kinase
RNAi	RNA interference
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBB	4,5,6,7-tetrabromobenzotriazole
TGFα	Transforming growth factor alpha

Chapter 1: Introduction

1.1 Glioblastoma

In 2006, brain tumours will account for an estimated 1.6% of new cancer cases diagnosed and 2.3% of cancer-related deaths in Canada (Canadian Cancer Society, 2006). Eighty percent of these brain tumours arise in adults between the ages of 50 and 60, and they are the most common form of solid tumour found in children (BC Cancer Agency, 2003).

Glioblastoma, a stage 4 astrocytoma, is the most aggressive form of brain tumour and patients have a median survival time with treatment of 9-12 months. Glioblastomas can be classified as secondary glioblastomas, which progress from lower grade tumours, or as primary or *de novo* glioblastomas, which develop without evidence that they have progressed from lower grade astrocytomas.

Treatment options for glioblastoma are very limited. Surgical removal of the complete tumour is generally impossible because of its invasiveness into neighboring tissue. Radiation treatment is a viable option that can improve survival rates (Yuile et al, 2006), but must be strictly controlled to minimize damage to healthy brain tissues, especially around the brain stem. Chemotherapeutic options are also limited. Currently, the drug temozolomide is often used in combination with radiotherapy as the most effective method of treatment (Stupp et al, 2005; Athanassiou et al, 2005). However, the frequency of recurrence for glioblastoma is very high, and exceedingly few patients remain alive and functional two years after diagnosis.

It is because of the inescapable outcome of the malignancy that a great deal of research has gone into studying glioblastoma. One important line of research has found the

epidermal growth factor receptor (EGFR) to be critically involved in the aggressiveness of a subset of primary glioblastomas (Reviewed in Ohgaki, 2005).

1.2 EGFR

Approximately 50% of glioblastomas have EGFR gene amplification (Libermann et al, 1985). EGFR, encoded by the *c-erbB1* gene (7p11-13, chromosome 7), is a transmembrane receptor protein that promotes cell growth and cell survival. Gene amplification of the receptor is thought to provide a means of increasing cell sensitivity and growth response to lower levels of ligand (Merlino et al, 1984).

EGFR belongs to the Type I receptor tyrosine kinase family of proteins, including EGFR (ErbB1), ErbB2 (HER2, neu), ErbB3 (HER3), and ErbB4 (HER4). This family of transmembrane receptor proteins binds at least 11 different ligands, which may be secreted and bound in either an autocrine or paracrine manner. Of the ligands, EGFR has the highest affinity for EGF and TGFα, but also binds amphiregulin, epiregulin, betacellulin, and heparin binding-EGF. The extracellular portion of EGFR is divided into 4 subdomains. Subdomains I and III support ligand binding, while subdomains II and IV serve structural roles (reviewed in Citri and Yarden, 2006).

Following binding of ligand to EGFR, the receptor undergoes dimerization. Dimerization occurs as a result of conformational changes in structural subdomains II and IV following ligand binding. These changes promote the exposure of a region on subdomain II called the "dimerization loop", which is the key mediator of the intermolecular interactions between the receptor pairs (Ogiso et al, 2002; Dawson et al, 2005). This loop is otherwise involved in intramolecular interactions between subdomains II and IV that prevent the loop

from binding to other ErbB molecules (Ferguson et al, 2003). This means that molecules of EGFR will not dimerize unless bound to a ligand (Citri and Yarden, 2006). EGFR may form dimers in any combination with ErbB family members (i.e. both homo- and hetero-dimers may form).

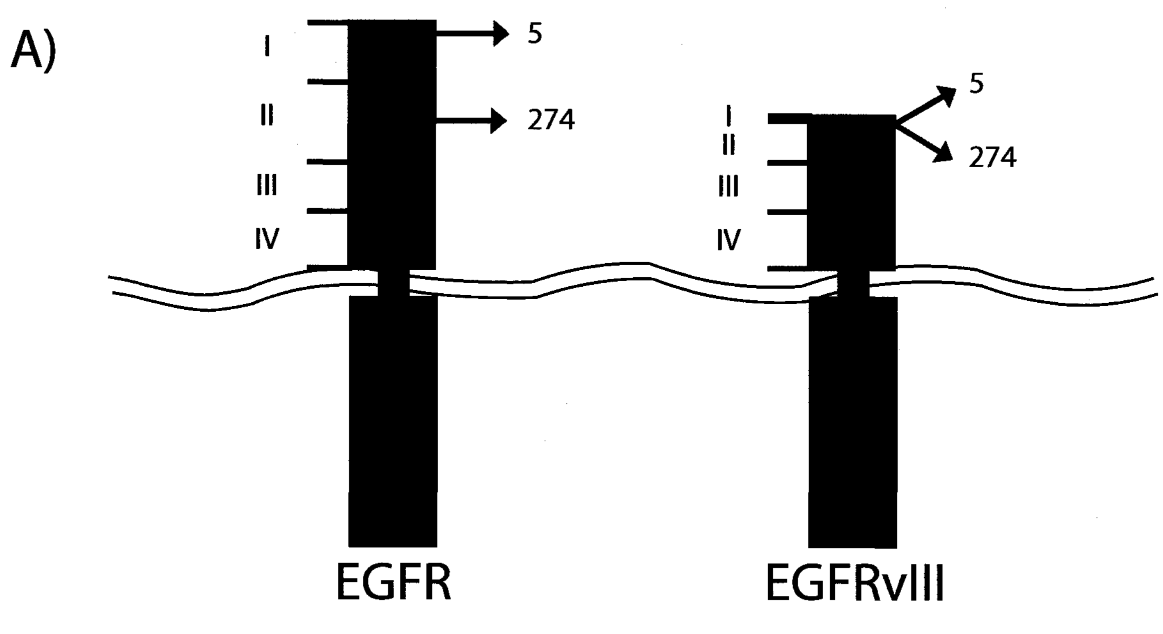
After dimerization, the tyrosine kinase domain on the intracellular portion of the receptor becomes activated, promoting *trans*-autophosphorylation of selected residues on its dimerization partner, and activation of downstream signaling. This signaling utilizes pathways including Ras and PI3K to promote cell proliferation, invasion, angiogenesis, and chemoresistance, creating a more robust, faster growing cell (Reviewed in Normanno et al, 2004).

1.3 EGFRvIII

Several types of EGFR mutant variants arise during EGFR gene amplification in brain tumours. Of these mutants, a mutation known as EGFRvIII (for variant III) is the most common and is seen in over 50% of cases with EGFR amplification (Bigner et al, 1990; Moscatello et al, 1995). The EGFRvIII gene has a deletion of exons 2-7, resulting in an 801bp in-frame deletion in the mRNA product. The EGFRvIII protein retains its membrane localization sequence, but lacks a ligand binding domain and one of its extracellular structural domains (Figure 1). Due to partial deletions in subdomains I and II, EGFRvIII can no longer bind ligand, but retains a weak ability to dimerize (Huang et al, 1997). The resulting protein has a constitutively active tyrosine kinase domain, which activates signaling pathways differently than wild-type EGFR (Chu et al, 1997; Lorimer et al, 2001).

Figure 1: Structural features of proteins EGFR, EGFRvIII, Hsp90, and Cdc37. EGFR and EGFRvIII are transmembrane proteins with their tyrosine kinase and C-terminal tail domains extending into the cytoplasm. The C-terminal tail contains all 5 autophosphorylation sites. EGFRvIII contains a deletion of amino acids 6-273 in its N-terminal extracellular domain, which prevents it from binding ligand. In EGFRvIII, amino acids 5 and 274 flank the deleted segment, which is replaced by a single glycine residue. This results in a loss of most of subdomain I, part of subdomain II, and 4 of the 12 N-linked glycosylation sites for on the mature receptor. B) Representation of the Hsp90 dimer. The major feature of the N-terminal domain is its ATPase domain and cochaperone binding ability. The middle domain is primarily responsible for client and cochaperone binding, while the C-terminus mediates Hsp90's dimerization and has a secondary, less characterized ATPase domain. C) Representation of Cdc37. Cdc37 interacts with N-terminal domain of Hsp90 via its middle and C-terminal portion. Binding to client is mediated by Cdc37's N-terminal domain, which contains a CK2 phosphorylation site on Serine 13. Serine 13 is the only phosphorylation site on Cdc37.

A)



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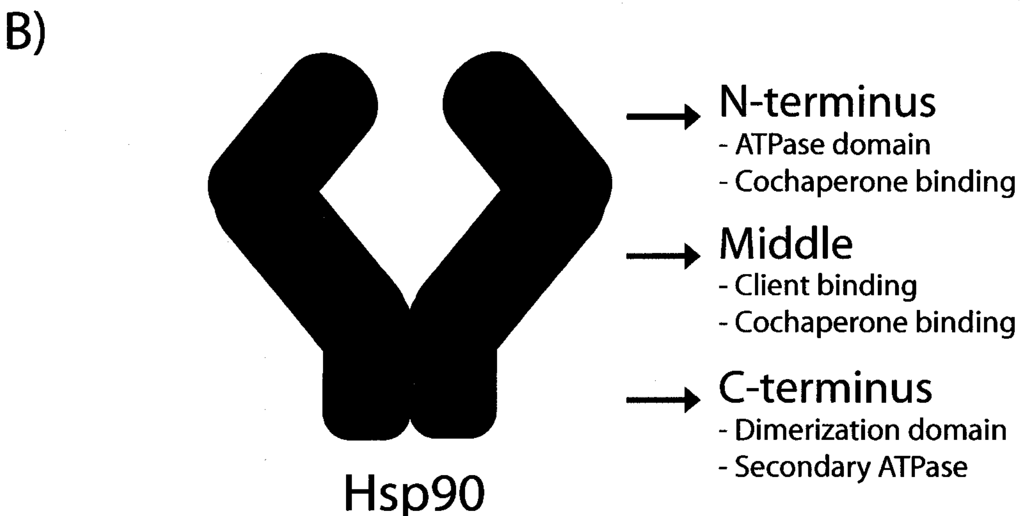

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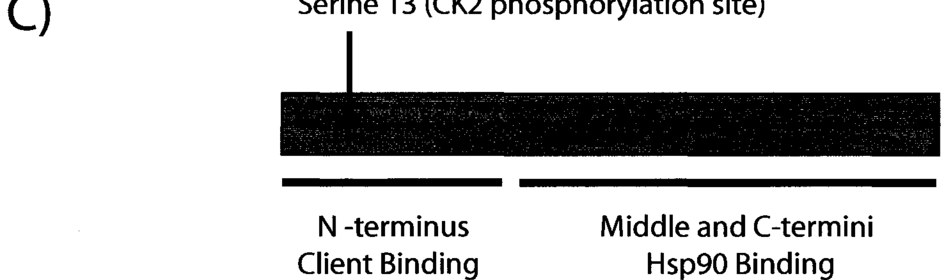


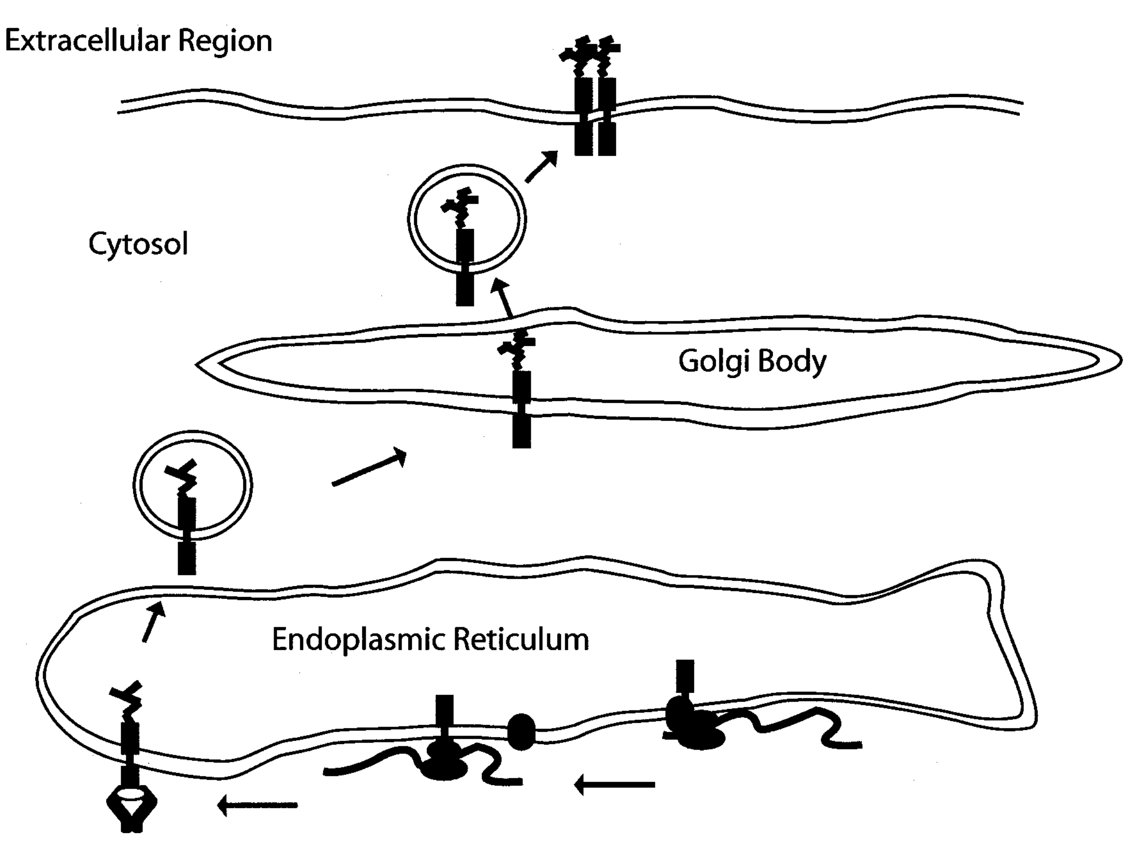
Furthermore, the internalization rate of mutant EGFRvIII following activation is reduced, resulting in enhancement of the signal (Huang et al, 1997). This signaling promotes increased proliferation, angiogenesis, and survival for the tumour and is independent of extracellular influence (Tang et al, 2000; Mishima et al, 2001, Montgomery et al, 2000).

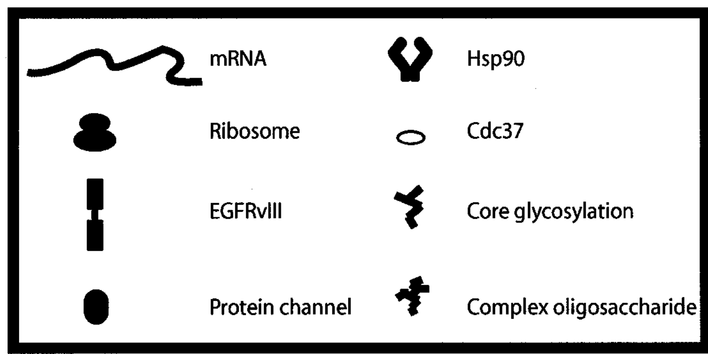
EGFRvIII has also been found in patients with carcinomas of the breast, lung, and ovary (Silva et al, 2006; Wikstand et al, 1995; Okamoto et al, 2003; Moscatello et al, 1995). Its expression is linked to poor prognosis in the subset of glioblastoma patients that survive for more than one year (Heimberger et al, 2005), and contributes to overall lower survival in glioblastoma cases with detectable EGFR amplification (Shinojima et al, 2003). Due to its role in such aggressive tumours, much research has gone into understanding EGFRvIII to assist in development of new therapeutic strategies.

1.4 EGFRvIII Biogenesis

EGFRvIII, like EGFR, is synthesized on the rough endoplasmic reticulum as a transmembrane protein (Figure 2). The truncated N-terminus of the protein resides within the ER, while the C-terminal kinase domain extends into the cytoplasm (Johns et al, 2005). In the ER, the chaperone proteins BiP and Grp94 interact with EGFRvIII's N-terminus, presumably to assist the stability of the nascent protein (Lavictoire et al, 2003). The N-terminus is also modified via N-linked core glycosylation at 8 separate sites while inside the ER. Nascent EGFRvIII is then transported to the Golgi apparatus where complex oligosaccharides are added to modify the core glycosylation motifs. From the Golgi, EGFRvIII is transported to the cell membrane as a mature protein, which may form dimers,

Figure 2: Biogenesis of EGFRvIII.
Translation of EGFRvIII occurs on the rough endoplasmic reticulum where a protein translocation channel protein likely assists in the insertion of the N-terminal domains into the ER. After translation, core glycosylation of N-terminal domain occurs within the ER lumen, while Hsp90 and cochaperone Cdc37 bind the cytoplasmic, C-terminal tail of EGFRvIII. Once complete, nascent EGFRvIII is be shipped in vesicles to the Golgi body where it is further modified through addition of complex oligosaccharides. It is unknown if association with Hsp90 continues at this point. Following passage through the Golgi, EGFRvIII is placed on the outer cell membrane, where it may dimerize, allowing for trans-autophosphorylation, and downstream signaling.


Extracellular Region
Cytosol
Golgi Body
Endoplasmic Reticulum


mRNA
Hsp90
Ribosome
Cdc37
EGFRvIII
Core glycosylation
Protein channel
Complex oligosaccharide

autophosphorylate, and activate downstream signaling molecules. It is the mature form of EGFRvIII that is the dominant form found in cells (Wikstrand et al, 1997).

1.5 EGFRvIII and Hsp90

In 2003, our research group published a report that identified an interaction of Hsp90 with the nascent form of EGFRvIII (Lavictoire et al, 2003). It was found that treatment with Hsp90 inhibitors decreased the expression of EGFRvIII in glioblastoma cells. Additionally, nascent EGFRvIII copurified with Hsp90 and Hsp70, their ER homologues Grp94 and Bip, as well as the Hsp90 cochaperone Cdc37. Together, these findings suggest that nascent EGFRvIII is a client protein of Hsp90 and is unable to mature without support from the chaperone system. Further understanding of this interaction may provide new avenues for targeting of EGFRvIII in glioblastoma.

One important innovation from our previous study was the discovery that a Flag-tagged EGFRvIII protein could be used to selectively purify nascent EGFRvIII. The tagged EGFRvIII expressed at the same level as an unmodified EGFRvIII construct, and showed identical levels of tyrosine kinase activity. However, the immunoreactivity of the N-terminal triple Flag epitope was only detectable in the endoplasmic reticulum and not on the cell membrane (Figure 2, Flag tag is not detectable within or after passage through the Golgi). By Western blot, tagged-EGFRvIII had a molecular weight that was slightly higher than untagged EGFRvIII. Further analysis of the Flag epitope suggested that it was covalently modified during trafficking in the Golgi, which rendered it undetectable by the anti-Flag antibody. This behavior of the Flag epitope was exploited to selectively study the pool of nascent EGFRvIII (Lavictoire et al, 2003).

1.6 Hsp90

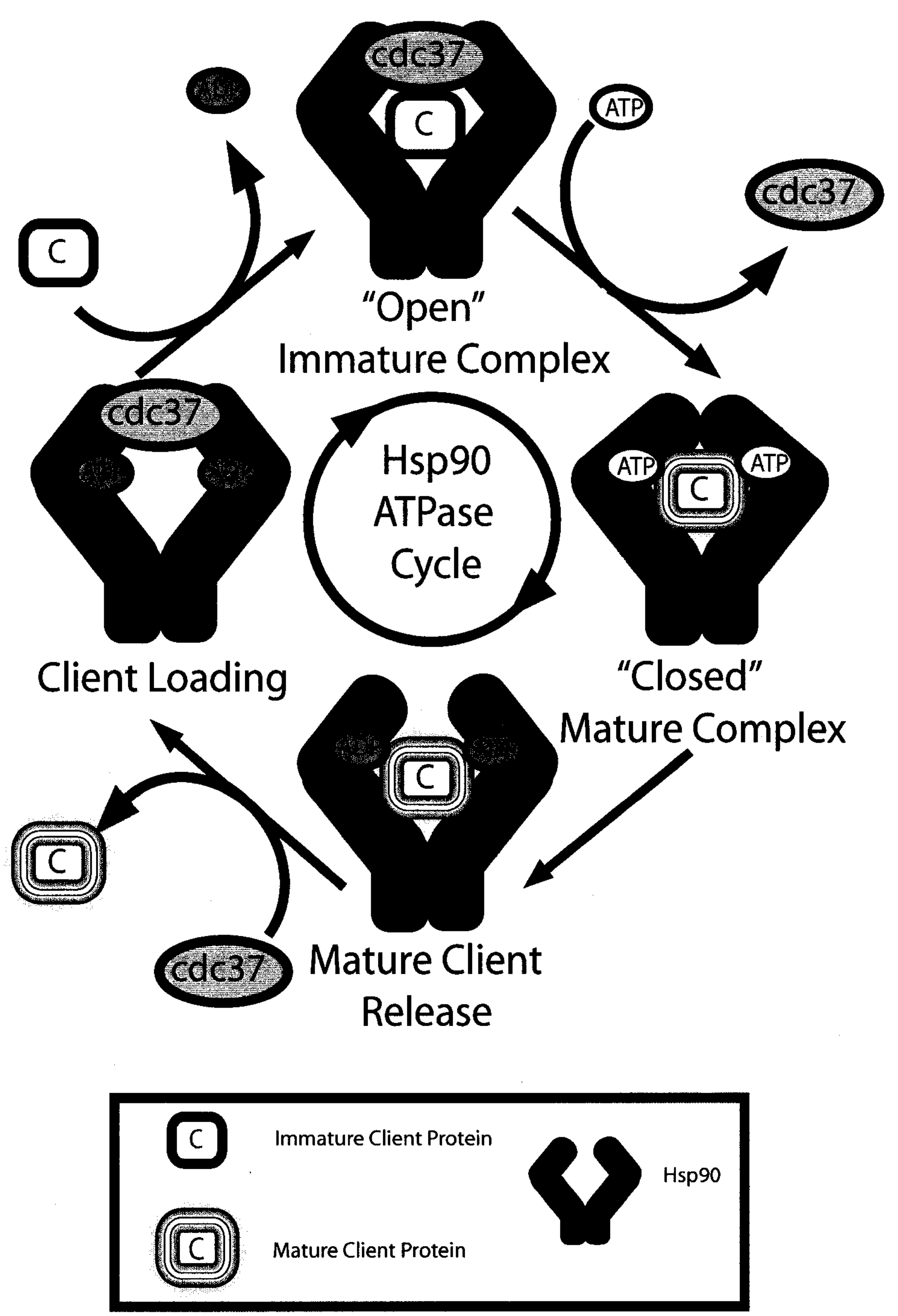
Hsp90 is a stress-inducible 90KDa molecular chaperone. Found constitutively expressed in the cytoplasm of all cells types, Hsp90 functions to assist in the folding of a large, but selective set of proteins, known as client proteins. While it can compose up to 1-2% of total protein in normal cells, Hsp90 levels can increase a further two-fold during stress (Lai et al, 1984).

Hsp90's function as a chaperone relies upon its formation of homodimers. The action of the Hsp90 dimer is commonly described using the "molecular clamp" hypothesis. By this hypothesis, the flexible structure of the Hsp90 dimer resembles a clamp that closes around client proteins to provide a controlled folding environment (Figure 3). The opening and closing of the Hsp90 chaperone clamp is driven by ATP binding and hydrolysis, and the entire process is regulated by Hsp90-interacting proteins termed "cochaperones", which control all aspects of Hsp90 activity, including ATP hydrolysis, client binding and release, and the conformational switching of Hsp90 (Prodromou et al, 2000).

1.7 Hsp90 Structure-Function Relationship

Structurally, Hsp90 consists of 3 major domains: N-terminal, middle, and C-terminal, all of which are necessary for its function (Figure 1). The N-terminal domain contains a major ATPase subdomain, which provides energy that is essential to the "molecular clamp" action in the chaperoning cycle (Figure 3). In its nucleotide-free state, the N-terminal domain is in an open conformation, permitting client binding. After a client has been complexed with Hsp90, ATP is recruited, and the N-terminal domain closes over the client protein,

Figure 3: Overview of the Hsp90 ATPase cycle for Cdc37 clients.
Hsp90's chaperone function is dependent upon its ability to use ATP to drive conformational changes that mediate client binding and release to the Hsp90 dimer. Clockwise from the left: The Hsp90 dimer in its "client loading" phase is considered to be in an "open", receptive conformation with ADP bound. Association of the cochaperone Cdc37, which recruits certain protein kinase clients, at this phase locks Hsp90 in its open form, and may help to directly mediate the recruitment of client to Hsp90. Following binding of an immature client protein, ATP is recruited, which causes the closing of the N-terminal portions of the Hsp90 dimer, creating a pocket for client folding. During this phase, Cdc37 is displaced from the complex by an unknown mechanism. In this closed conformation, the client protein may fold into its intended, mature form, and is released from the complex when Hsp90 hydrolyzes the bound ATP into ADP. ATP hydrolysis triggers the Hsp90 dimer to reopen, expel the mature client, and reassume its receptive conformation.


cdc37
C
ATP
cdc37
C
"Open"
Immature Complex
cdc37
Hsp90
ATPase
Cycle
ATP
C
ATP
Client Loading
"Closed"
Mature Complex
C
C
cdc37
Mature Client
Release
C
Immature Client Protein
Hsp90
C
Mature Client Protein

forming a pocket in which the client may fold. Recently, it was determined by examining crystal structures of a yeast Hsp90 complex in a closed conformation that the Hsp90 dimer does not close fully around its client protein as previous thought (Ali et al, 2006). Instead, the closed dimer creates a pocket which provides the folding environment for the client protein. The length of time that Hsp90 remains closed is conditional, likely under strict regulation by cochaperones, like p23, and dependent upon the nature of the client. The final stage of the ATPase cycle comes when ATP is hydrolyzed, and the N-terminal domains of the Hsp90 complex open, allowing for release of the client protein. The movement of the N-terminal domain is permitted by a flexible linker region which separates it from the middle domain.

The middle domain of Hsp90 plays a role in mediating interactions with both client proteins and select cochaperones (Meyer et al, 2003). Due to overlap of binding sites, only certain combinations of cochaperones may be bound at any one time. The middle domain contains a tetratricopeptide repeat domain, used for interaction to a subset of cochaperones (Cliff et al, 2006). These tetratricopeptide repeat domain cochaperones include the immunophilins, Hop and PP5 (Smith, 2004). Other cochaperones interact with other, specific sites on the protein.

Hsp90 is found in the cytoplasm primarily as a homodimer. Dimerization is mediated through the C-terminus of the protein and is required for Hsp90 chaperone activity. A second, weaker, but necessary ATPase domain has been reported on the C-terminus; however its specific role in regulating the chaperone cycle is unknown (Marcu et al, 2000).

1.8 Hsp90 and Cancer

Although ubiquitously expressed in all cells, Hsp90 is overexpressed in many tumours, where it also has an activity about 40 times higher than in normal cells (Kamal et al, 2003). It was also shown that Hsp90 found in tumour cells is almost entirely active, and found in cochaperone complexes, whereas Hsp90 in normal cells is in an inactive, uncomplexed form. This suggests that inhibitors that target Hsp90 in its active form should have tumour specific activity. Kamal *et al* proposed that the increased expression and activity of Hsp90 in tumours allows them to cope with increasing numbers of overexpressed and mutated signaling proteins as the tumour progresses. Another factor may be that more active Hsp90 is required to help these cells maintain proper protein form and function amid the many stresses of the tumour micro-environment, which include ischemia, acidosis, nutrient deprivation, and cytotoxic drug treatments.

Hsp90 is considered a promising drug target against cancer because it assists in the expression of many oncogenic client proteins. These clients include EGFR, EGFRvIII, ErbB2, Akt, Raf-1, Erk1/2, Cdk4, Bcr-Abl, and mutant p53 (Xu et al, 2001; Stancato et al, 1997; Zhang et al, 2005; Setalo et al, 2002; Yu et al, 2002). By stabilizing or assisting in the activation of these proteins, Hsp90 improves their capacity to function within the cell, and enhances their tumourigenicity.

Along with its role in stabilizing oncoproteins, Hsp90 may also act as a capacitor for evolution in cancer. *Drosophila* studies have found that partial inhibition of Hsp90 causes large-scale phenotypic variation in flies (Rutherford and Lindquist, 1998; Queitsch et al, 2002; Sollars et al, 2002). This has been attributed to Hsp90's ability to silence the effects of protein variants by guiding them towards stable conformations with wild-type activities.

Promoting wild-type function of mutant proteins permits genetic drift and heterogeneity within the tumour cell population. This population diversity may make a tumour more robust, as acquired mutations may provide a survival advantage under selective conditions. Thus, ablating Hsp90 activity may result in destabilization of proteins that could help a tumour cope with the selection conditions provided by radio- and chemotherapeutics.

Experimentally, the drug geldanamycin (GA) has shown high molecular selectivity for Hsp90 and the ability to inhibit Hsp90 activity at nanomolar concentrations (Gooljarsingh et al, 2006). Geldanamycin binds to the ATP binding site on the N-terminal ATPase domain of Hsp90, inhibiting its function, and leading to degradation of client proteins. Clinically, however, GA has shown severe hepatotoxicity, attributed to a product of its degradation, and not Hsp90 inhibition (Eiseman et al, 2005; Page et al, 1997). Due to this failing, a second generation drug, known as 17-allylaminogeldanamycin, was designed to improve GA's stability and bioavailability (Reviewed in Sharp and Workman, 2006). Currently, 17-AAG is undergoing phase II clinical trials, showing promise against advanced refractory cancers, including prostate cancer, melanoma, and multiple myeloma (Heath et al, 2005, Banerji et al, 2005, Duus et al, 2006).

1.9 Hsp90 Cochaperones

Hsp90 cochaperones are non-client interactors that regulate Hsp90's function by controlling its progression through the ATPase cycle. Most of the cochaperones slow Hsp90's ATPase activity, which is thought to improve the efficiency of client recruitment and folding. Over a dozen cochaperones are known to exist, each having a relatively unique and specific function. Some of the most studied cochaperones include Hsp70, p23, Hop,

Aha1, CHIP, Hsp40, Cdc37, and the immunophilins FKBP51 and FKBP52. This list can be loosely divided into early and late-stage cochaperones, depending on the stage of the Hsp90 ATPase cycle during which they interact with Hsp90. Although the components of the pathway vary from client to client, the roles of each particular cochaperone appear to be consistent. In general, the first step of the chaperoning pathway involves the binding of Hsp40 to Hsp70, which activates Hsp70 and stimulates its ability to bind client (Fan et al, 2003). Hop then acts as a scaffold between Hsp70 and the Hsp90 dimer, which results in the transfer of client to Hsp90 (Johnson et al, 1998). At this point, Hsp90 binds ATP, and is recognized by p23, whose binding appears to cause dissociation of the early stage cochaperones Hsp70, Hop, and Hsp40 (Sullivan et al, 2002). P23, binds to the middle domain of the ATP-bound, closed-conformation form of Hsp90, and stabilizes this state, extending the timeframe for client folding, while slowing ATP turnover (Morishima et al, 2003). At this point, another cochaperone, Aha1, which also binds to the middle domain of Hsp90, displaces p23 from the late-stage Hsp90-client complex and promotes ATP hydrolysis and client release from the complex (Harst et al, 2005).

Although numerous cochaperones have been identified, there are still many questions remaining about their function. Little is known about the triggers that induce binding and release of cochaperones to and from the Hsp90 complex. As well, the cochaperones involved in folding of an Hsp90 client appear to vary between client proteins. This means that the requirement for a cochaperone may vary depending upon the particular needs of the client, which cannot be predicted by our current knowledge of the system.

With respect to EGFRvIII, we have previously identified Hsp90, Hsp70 and the cochaperone Cdc37 as cytosolic interactors with the nascent form of the protein (Lavictoire

et al, 2003). Cdc37 is an early stage cochaperone that is directly responsible for the recruitment of many protein kinase clients, including many oncogenic proteins, to the Hsp90 complex. Cdc37 has also been shown to bind ErbB2, as well as an EGFR protein mutated within its kinase domain, and is speculated to control their differential recruitment to Hsp90 (Xu et al, 2005). However, the role of Cdc37 in the chaperoning of these clients, or any other protein synthesized on the ER, has not yet been examined.

1.10 Cdc37

Cdc37 is a cochaperone protein with a principal role in the recruitment of certain protein kinases to the Hsp90 complex. The specificity of Cdc37 for kinases makes it unique among the cochaperones, and has attracted interest from the anti-cancer field.

1.11 Cdc37 in Cancer

Aside from its role as a regulator of the cell division cycle in yeast (Gerber et al, 1995), Cdc37 also appears to have an important role in mammalian tumours. The human homologue of Cdc37 is a 50 kDa protein that has a role in recruiting many important oncogenic protein kinases to the Hsp90 chaperone complex. Cdc37 overexpression has been linked to prostate tumour formation in humans and mammary tumour formation in mice, where it is believed it normally exists as a rate-limiting factor in the activity of its oncogenic clients (Schwarze et al, 2003; Stepanova et al, 2000). The list of known Cdc37 client kinases involves many important proteins involved in cell proliferation, including Cdk4, Aurora B, v-src, Raf1, Akt, MOK, CK2, ErbB2, and EGFRvIII (Miyata and Nishida, 2004; Bandhakavi et al, 2003; Xu et al, 2005; Lavictoire et al, 2003; reviewed in Pearl, 2005). Although

evidence suggests that its primary role is its function as an Hsp90 cochaperone, its ability to partially compensate for lack of Hsp90 in yeast suggests possible Hsp90-independent functions that should not be overlooked (Rao et at, 2001; Kimura et al, 1997).

1.12 Cdc37 in the Hsp90 Chaperone Cycle

Cdc37 functions as an adaptor protein, promoting the interactions between Hsp90 and client protein kinases (Figure 3). This is mediated through separate interactions of two of its major structural domains (Figure 1) (Reviewed in Pearl, 2005; Prodromou and Pearl, 2003). While its N-terminal domain binds client proteins, Cdc37 uses its middle and C-terminal domains to directly bind to the N-terminus of Hsp90. This interaction maintains Hsp90 in its "open", receptive conformation and temporarily arrests ATPase activity (Siligardi et al, 2004). It is believed that by locking Hsp90 in this receptive conformation, Cdc37 can more efficiently recruit its specific subset of client proteins to the chaperone complex. While Cdc37 stops the ATPase activity of Hsp90, this does not interfere with the binding of nucleotides, or inhibitors, to the ATPase domain of Hsp90 (Siligardi et al, 2002). Thus, Cdc37 may render Hsp90 more susceptible to ATPase inhibitors by giving them an extended window in which to bind. This means that client proteins recruited by Cdc37 may be more sensitive to Hsp90 inhibitors than other client proteins (Siligardi et al, 2004).

Following recruitment of a kinase client protein, Cdc37 dissociates from the complex, allowing the Hsp90 clamp to close, and permit Hsp90 and other cochaperones to facilitate stabilization of the protein. It is not well understood what causes the release of Cdc37 from the Hsp90 complex after client binding; however the recruitment of late-stage

chaperones, such as p23, which bind in a mutually-exclusive manner with Cdc37, may be responsible for its displacement (Harst et al, 2005).

1.13 Cdc37 Binding to Client Proteins

The binding of Cdc37 to client proteins is largely mediated by its N-terminal domain. While some researchers have tried to find a common Cdc37-binding motif shared among its clients, it appears that no such conserved amino acid sequence can be universally defined (Terasawa et al, 2006; Prince et al, 2005; Prince and Matts, 2005). Identifying these interaction motifs is particularly difficult because Cdc37 interactions are extremely selective, discriminating between members of highly homologous kinase families. Instead, it is most likely that Cdc37, like Hsp90, identifies specific properties of exposed surfaces on clients (Citri et al, 2006). The shared properties of these surfaces, however, have not yet been defined.

Whether Cdc37 first binds to the client protein or Hsp90 is debated. Complexes containing only Cdc37 and Cdk4 have been identified, suggesting that client binding may occur independently of Hsp90 (Vaughan et al, 2006). However, treatment of cells with geldanamycin was shown to prevent both Cdc37 and Hsp90 from interacting with the client eIF2 alpha kinase (Shao et al, 2001). This geldanamycin sensitivity was lost on a C-terminally truncated form of Cdc37, which could not bind Hsp90 but could still bind client. This suggested a strong regulatory role for Hsp90 on controlling Cdc37 client binding.

1.14 Cdc37 Serine 13 Phosphorylation

The amino acids nearest the N-terminus of Cdc37 are highly conserved and are crucial for client binding (Fliss et al, 1997). Specific attention has been paid in recent years to a serine residue in this domain whose phosphorylation may be required for client binding to take place. The importance of this site was first recognized in a strain of yeast expressing a mutant Cdc37 protein that significantly slowed proliferation (Fliss et al, 1997). The mutation responsible was recognized as a Ser-14 to Leu-14 mutation, equivalent to Ser-13 in mammals. At this time, there are only a small number of publications concerning the role of serine-13 phosphorylation in mammalian Cdc37. These are summarized in the following.

One study by Shao *et al.* focused on evaluating the necessity and impact of Cdc37 phosphorylation on its ability to interact with Hsp90 and client proteins (Shao et al, 2003). They used an *in vitro* approach using rabbit reticulocyte lysates expressing the Hsp90/Cdc37 client heme-regulated eIF2-α kinase (HRI) and various mutant forms of Cdc37. A S13A Cdc37 mutant was created to examine the function of a non-phosphorylated Cdc37. This mutant had a 98% lower binding affinity for HRI than wild-type Cdc37 and a 40% lower affinity for Hsp90. A S13E mutant was also studied as a possible phospho-mimetic Cdc37, based on the similar steric and electronic properties between the carboxylate anion of glutamic acid and phosphorylated serine. This mutation showed an 85% decrease in binding to HRI, and a 10% lower affinity to Hsp90. These data were taken to mean that phosphorylation of Ser-13 is critical for binding of Cdc37 to HRI, and confirmed that a S13E mutant could partially mimic the binding of a pool of wild-type Cdc37 (Sricharan et al, 2003).

The same group also identified the serine-13 phosphorylation site as a CK2 phosphorylation motif and showed using purified proteins that CK2 could successfully phosphorylate this site (Shao et al, 2003). In this case, matrix-assisted laser desorption/ionization – time of flight mass spectrometry was used to experimentally identify the phosphorylation site on Cdc37. Additionally, they found that the wild-type Cdc37 in their rabbit reticulocyte lysates and in K562 human leukemia cells was almost entirely in its phosphorylated, active form.

In another study, Arlander et al. reported that the protein Chk1 is a client protein of Hsp90 and Cdc37 (Arlander et al, 2006). In their report, they sought to determine the minimum chaperone components necessary for stabilization and activation of Chk1. Using purified proteins in an *in vitro* model, they determined that the reaction required both Cdc37 and CK2 in addition to other chaperone components, and that a S13A Cdc37 mutant could not compensate for wild-type Cdc37 in the presence of CK2.

Two other studies in the field were performed by Miyata and Nishida that elaborated upon the importance of the Cdc37 / CK2 interaction (Miyata and Nishida, 2004; Miyata and Nishida, 2005). After showing a direct association between Cdc37 and the catalytic subunit of CK2, they demonstrated that CK2 phosphorylates Cdc37 only on serine-13, and that this phosphorylation is lost when cells are treated with the CK2 inhibitor, TBB. They found that treatment of cells with TBB destabilized the Cdc37 client proteins MOK and Raf-1, but not non-client proteins. Furthermore, they reported that relative to wild-type Cdc37, a S13A Cdc37 mutant had a decreased binding affinity to Hsp90 and client proteins Akt, Cdk4, v-Src, Aurora-B, MAK, MRK, MOK, and Raf-1.

Most recently, a study looking at the interaction of Cdc37 with Cdk4 noted that Cdc37, which co-purified with the Cdk4/Hsp90 complex, was phosphorylated on serine 13, and was resistant to phosphatase treatment inside this complexed form (Vaughan et al, 2006).

Taken together these data agree that phosphorylation of serine 13 on Cdc37 is essential for client protein binding, and that CK2 is likely the only kinase able to phosphorylate this site. This suggests a novel therapeutic strategy for selectively targeting the many oncogenic client proteins of Cdc37 in cancer.

1.15 CK2

CK2 is a ubiquitously expressed, highly conserved pleiotropic serine/threonine kinase that consists of two different catalytic α/α' subunits and two identical regulatory β subunits (reviewed in Litchfield, 2003). The catalytic subunits of CK2 have constitutive activity and are positively regulated via the stabilizing interactions with their regulatory β subunits. CK2 is a truly pleiotropic kinase with over 100 known physiological substrates and is not confined to any one compartment in the cell, providing a further level of complexity to its study. Regulation of CK2 is somewhat unclear and seems to be independent of second messengers or phosphorylation events. Although its molecular functions are not precisely defined, CK2 is known to promote cell proliferation, regulate cell death, and is required for growth and development in mice and C. *elegans* (Guerra and Issinger, 1999).

CK2 protein levels appear to be tightly regulated in normal cells but are upregulated in numerous tumour types, including breast, lung, prostate, and head and neck (Guerra and Issinger, 1999). Therapeutics have been designed with CK2 in mind. Most notably, an anti-

sense oligodeoxynucleotide specific to CK2 α has shown to be highly effective in mouse xenograft model with little effect on normal cells, both *in vitro* and *in vivo* (Ahmad et al, 2005). For experimental purposes, several chemical CK2 inhibitors exist; mostly these are competitive inhibitors for the ATP binding site on the catalytic subunits. Of these, TBB is thought to be the most specific and is commonly used as a CK2 inhibitor in studies on cultured cells (Sarno et al, 2005).

1.16 Hypothesis

Our lab has shown that the molecular chaperone Hsp90 interacts with nascent EGFRvIII, and that EGFRvIII expression is dependent upon Hsp90 activity. In tumours, Hsp90 is found exclusively in an active form, where it is bound to cochaperones. Of these cochaperones, we found Cdc37 in a complex with EGFRvIII and Hsp90. I hypothesize that Cdc37 may control EGFRvIII biogenesis by regulating its recruitment to Hsp90. Since phosphorylation of Cdc37 is necessary for its recruitment of client proteins to Hsp90, it may also be a means by which to control EGFRvIII stability.

1.17 Specific of Objectives

1) Determine the role for Cdc37 in EGFRvIII biogenesis
2) Determine the importance of Cdc37 phosphorylation in EGFRvIII biogenesis
3) Determine if inhibiting CK2 activity affects the stability of EGFRvIII and its recruitment to Cdc37

Chapter 2: Materials and Methods

2.1 Cell lines

The human glioblastoma cell line U87MGtftΔ, which expresses triple-Flag tagged EGFRvIII, was created as described previously (Lavictoire et al., 2003). All cells were cultured in 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium supplemented with 2mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% serum (3:1 v/v mixture of donor bovine serum and fetal bovine serum).

2.2 Antibodies and Reagents

Mouse monoclonal antibody for Cdc37 was obtained from Affinity Bioreagents. Rabbit polyclonal antibody to Cdc37, as well as antibodies against EGFRvIII (Ab-12) and Cdk4 (Ab-6) were from Neomarkers. Antibodies against actin and Flag (M2) were from Sigma, while pan-ERK and phosphotyrosine (PY-20) were from BD Biosciences.

Geldanamycin was purchased from Calbiochem. Both 4,5,6,7-Tetrabromo-2-azabenzimidazole (TBB) and proteasome inhibitor cocktail were obtained from Sigma. Custom DNA oligonucleotides were synthesized by Integrated DNA Technologies.

2.3 Mutagenesis of Cdc37

Serine 13 of Cdc37 was mutated in the pLPCX/Cdc37 WT vector to create non-phosphorylatable alanine and phosphomimetic glutamic acid mutant constructs. Mutagenesis of serine 13 on Cdc37 to alanine (Cdc37 S13A) and glutamic acid (Cdc37 S13E) was performed on pLPCX/Cdc37 WT using the QuikChange XL site-directed

mutagenesis kit (Stratagene). The following primer pairs were used for the respective mutations: S13A primers – fwd 5' GGGACCACATTGAGGTGGCTGATGATGAAGACGAGACGC 3', rev 5' GCGTCTCGTCTTCATCATCAGCCACCTCAATGTGGTCCC 3'; S13E primers – fwd 5' GGGACCACATTGAGGTGGAAGATGATGAAGACGAGACGC 3', rev 5' GCGTCTCGTCTTCATCATCTTCCACCTCAATGTGGTCCC 3'. Codons for the mutated amino acids are underlined. Both sets of primers were annealed at 60°C for 50 s followed by a 12 minute extension at 68°C. All other procedures for the mutagenesis are outlined by the kit. Positive mutants were confirmed by sequencing the Cdc37 cDNA insert using primers flanking the multiple cloning site of pLPCX (5' AGCTCGTTTAGTGAACCGTCAGATC 3' and 5' ACCTACAGGTGGGGTCTTTCATTCCC 3').

2.4 Transduction of Cdc37 Constructs

U87MGtftΔEGFR cells were stably transduced to express Cdc37 WT, Cdc37 S13A, Cdc37 S13E, or an empty vector control using a replication-incompetent MoMLV retroviral vector targeting the ecotropic receptor presented by these cells. These retroviruses were created using a three plasmid transfection technique (Soneoka et al., 1995). Briefly, this involved the mixture of 7 μg of three plasmids: pHIT 60 (*gag* and *pol* genes), pHIT123 (ecotropic *env*), and the pLPCX/Cdc37 contructs (Ψ packaging signal, amp resistance, inserted Cdc37 cDNA) with ddH$_2$O to a volume of 90 μL. DNA was then precipitated by addition of 10 μL 3M sodium acetate (pH 5.2) and 200 μL anhydrous ethanol, then chilling on ice. After 1 hour, the tube was spun at >15,000 x g for 10 minutes to pellet the DNA. Supernatant was carefully removed from the pellet, which was then allowed to dry for 10

minutes. The pellet was dissolved in 450 μL ddH_2O. The redissolved pellet was added to 50 μL of 2.5 M $CaCl_2$, then added dropwise, with mixing into 500 μL of 2X HeBs solution (42 mM HEPES, 2.8 mM Na_2HPO_4, 274mM NaCl, pH 7.1). This was left for 20 minutes at room temperature, then added to 293T cells plated the day before at $2x10^6$ per 10 cm plate in 10 mL medium. The following day, the media was removed from each plate, followed by a rinse with PBS (137 mM NaCl, 10 mM Na_2HPO_4, 2.7mM NaCl, pH 7.4) and addition of 10 mL of fresh media. Virus was rescued the next day by collecting the media from each plate, passing it through a 0.45 μm filter, then adding it in a 1:1 mixture with 5 mL 16 μg/μL polybrene. This was added to U87MGtftΔ cells plated the day before at $2x10^5$ cells per 10 cm dish. Virus-containing media was removed following 16 hours and replaced with standard media. Cells were permitted to grow for two days before being submitted to selection with 1 μg/mL puromycin. Selection was maintained for approx. 10 days. A mock transduction with virus-free 293T media was performed as a control for efficient selection.

2.5 Immunoprecipitation of Triple-Flag Tagged EGFRvIII

U87MGtftΔEGFR cells transduced to express Cdc37wt, Cdc37S13A, Cdc37S13E, or the empty vector control were plated at $6x10^6$ cells per 10 cm plate. A U87MG cell line expressing untagged EGFRvIII and Cdc37S13E was plated for use as a negative control. The next day, cells were rinsed twice with ice-cold PBS, then 600 μL IP buffer (10 mM Tris-HCl, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 2 mM Na_3VO_4, 0.5% NP-40, 0.2 mM PMSF, 1 mM EGTA, 1x Proteasome inhibitor cocktail, 0°C, pH 7.4) was added. Cells were collected and passed once through a 27-gauge needle, then insoluble debris was removed by centrifugation at 16,000 x g for 15 minutes at 4°C. 40μL of uniformly suspended Anti-Flag

resin was prepared for each sample by rinsing twice with 300 μL IP buffer, then suspended in 400 μL IP buffer for use. 600μL of soluble lysate was added the resin mixture, and incubated at 4°C for 2 hours on a rocking platform. Resin was pelleted by spinning at 8,000 x g for 30 seconds, then rinsed three times with 300 μL IP buffer. Bound proteins were eluted by adding 60μL of 2X Laemmli buffer (4% (w/v) sodium dodecyl sulfate, 0.1% glycerol, 125 mM Tris-HCl, 0.3 mM 2-mercaptoethanol, pH 6.8) and heating at 100°C for 5 minutes.

2.6 Geldanamycin Treatments

U87MGtftΔEGFR cells transduced to express Cdc37S13A or Cdc37S13E were plated at $2x10^5$ cells per well in 6 cm plate. The next day, cells were treated with either geldanamycin (300 nM or 500 nM) or DMSO control. After 16 hours, cells were harvested in lysis buffer. Protein levels were analyzed by Western blot.

2.7 TBB Treatments

U87MGtftΔEGFR cells transduced to express Cdc37wt or empty vector control were plated at $2x10^5$ cells per well in 6 cm plate. The next day, cells were treated with either TBB (60 μM or 120 μM) or DMSO control. After 16 hours, cells were harvested in lysis buffer for immunoprecipitation or Western blot.

2.8 Two-Dimensional Gel Electrophoresis

U87MGtftΔEGFR/ Cdc37 WT cells were plated at $2x10^6$ cells in a 10cm plate. After 24 hours, cells were rinsed with PBS and collected in 0.5mL sucrose buffer (0.3M sucrose). Cells were pelleted by centrifugation at 300 x g for 5 min, then resuspended in 0.5 mL IEF buffer (7 M Urea, 2 M Thiourea, 0.1 M CHAPS, 0.1 M DTT, 0.1% bromophenol blue). Undissolved particles were then removed by centrifuging at 12,000 x g for 2 minutes. Proteins were precipitated from solution by adding 5 mL acetone, and incubating for exactly 4 min at room temperature. Protein was pelleted by spinning at 1,000 x g for 5 min. Supernatant was discarded and the pellet was dried under vacuum for 2 min. The pellet was then resuspended in 0.3 μL IEF buffer + 50 μL biolytes (3/10, Biorad). This volume was pipetted along the edge of a disposable strip dish and a pH 4-7 IPTG strip (BioRad) was placed on top, covered in mineral oil, and allowed to absorb for 16 hours. The next day, excess oil was removed and the strip was placed in an isoelectric focusing chamber (BioRad) programmed for 1 hr at 200 V, 1hr at 500 V, 5 hr ramp to 5,000 V, 80,000 V hours focusing at 5,000 V, then maintained at 500 V until shut off. A urea solution was prepared (3.6 g Urea, 3.0 g glycerol, 0.92 mL 1.5 M Tris-HCl pH 8.8, fill to 10 mL dH_2O). The strip was then placed in reducing (5 mL Urea solution, 0.05 g DTT, 0.1 g SDS) and alkylating (5 mL Urea solution, 0.2 g iodoacetamide, 0.1 g SDS) solutions for 15 min each, then rinsed in running buffer and run on a 12% polyacrylamide gel. Proteins were transferred to a PVDF membrane, and Cdc37 protein was detected by traditional Western blotting techniques.

2.9 SDS-PAGE and Western Blot

Total cell lysates were collected in boiling-hot lysis buffer (100 mM Tris, 1% sodium dodecyl sulfate, 10% glycerol, pH 6.8). Lysates were heated at 100°C for 5 minutes, sonicated to disrupt protein-protein interactions, then heated again at 100°C for 5 minutes. Protein concentration was determined using the BCA Protein Assay Kit (Pierce). 80 μg of protein lysate was added to 2X Laemmli buffer (4% (w/v) sodium dodecyl sulfate, 0.1% glycerol, 125 mM Tris-HCl, 0.3 mM 2-mercaptoethanol, pH 6.8) for a total volume of 50 μL, then heated at 100°C for 5 minutes. Samples were loaded and run on NuPAGE 4-12% Bis-Tris polyacrylamide gels (Invitrogen) then transferred to PVDF membrane (Amersham) using the XCell II Blot Module (Invitrogen). Membranes were stained for total protein using amido black to ensure equal loading and transfer. Protein bands identified by Western blot were visualized using a GeneGnome (Gene Flow, UK) analysis instrument and quantified, where applicable, using Gene Tools software (Gene Flow).

2.10 Flow Cytometry

Cell cycle analysis was performed by flow cytometry using propidium iodide staining. Cells were plated at $2x10^5$ cells per 10 cm plate in complete media containing either 0.5 or 10% serum. Cells were collected the next day by treating with trypsin, pelleted by centrifugation at 3,000 x g for 5 minutes, and rinsed twice with PBS. Pelleted cells were then resuspended in 1mL ice-cold ethanol and allowed to fix for at least 2 hours at 4°C. Fixed cells were rinsed twice in PBS, pelleted, and resuspended in 1mL propidium iodide solution (50 μg/mL propidium iodide in PBS) plus 50 μL of 10 μg/mL RNase A. After at least 1 hour incubation at 4°C, stained cells were analyzed on a Beckman-Coulter Epics XL

(BD Biosciences). Data were analyzed using ModFit LT (Verity Software House, Topsham, Maine) to determine the status of the samples in the cell cycle. This experiment was performed using triplicate samples and was repeated twice. Statistical analysis of data was performed using two-tailed T-tests in Microsoft Excel.

Chapter 3: Results

3.1 Activation Status of Nascent Flag-tagged EGFRvIII

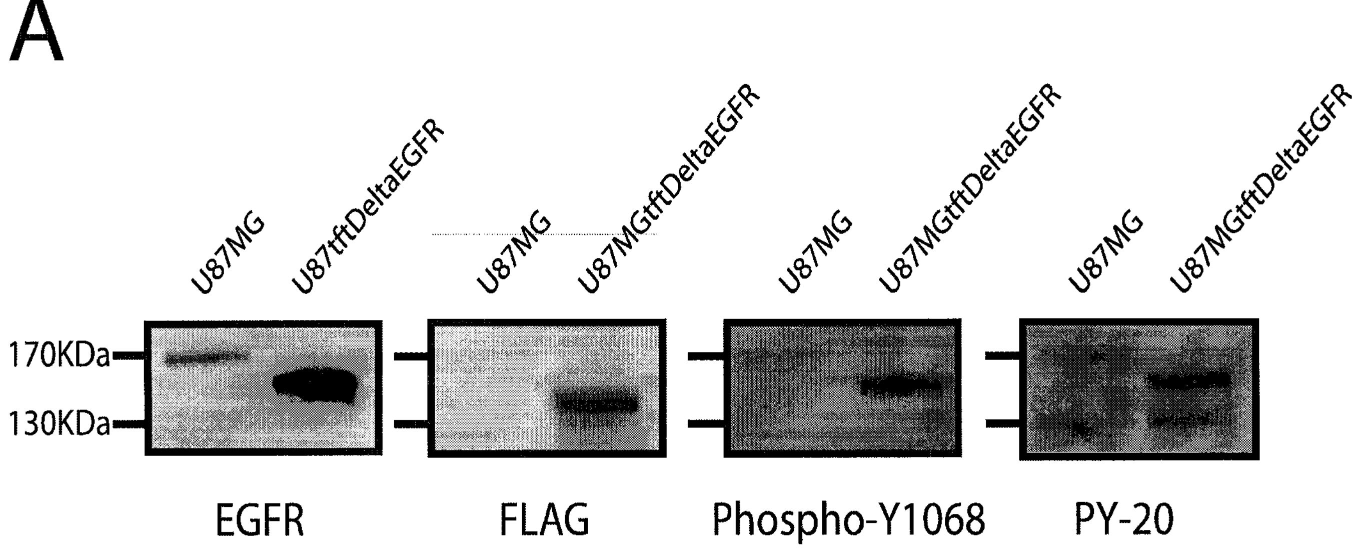
Our lab has previously demonstrated that the Flag epitope of triple-Flag tagged EGFRvIII (tftΔEGFR) is only detectable on the nascent form of the protein in the endoplasmic reticulum (Lavictoire et al, 2003). The epitope was covalently modified inside the Golgi such that it was undetectable by an anti-Flag antibody. Although the Flag epitope was modified, the level of expression and activity of the mature receptor was equivalent to that of untagged EGFRvIII.

It was found that EGFRvIII can be detected as two bands by Western blot. Analysis of EGFRvIII banding patterns following treatment with deoxymannojirimycin, an inhibitor of complex oligosaccharide formation, and tunicamycin, an inhibitor of core-glycosylation, showed that the major, upper band of the EGFRvIII doublet is the mature, fully processed form of EGFRvIII, while the minor, lower band is the immature, partially glycosylated form. The Flag epitope was only detectable in the lower-weight band of EGFRvIII, making it a specific marker for EGFRvIII that has been glycosylated, but lacks the complex oligosaccharides that are added within the Golgi. One thing that was not analyzed is the activation status of the nascent form of EGFRvIII. It is possible that a constitutively active mutant protein, like EGFRvIII, may have the ability to dimerize, autophosphorylate and begin signaling before reaching the cell membrane. Since this possibility was not examined, I first performed Western blot analysis on lysate from U87tftΔEGFR cells, looking for an indication of phosphorylated nascent EGFRvIII. Blots were probed with antibodies against EGFR, Flag, phospho-Y1068 (selectively detects phosphorylation of tyrosine 1068 on EGFR

and EGFRvIII), as well as PY-20, a general phosphotyrosine antibody. As a control for this experiment, I used lysate from the U87MG cell line, which expresses wild-type EGFR but not EGFRvIII. Two bands for EGFRvIII were detected, a major upper band, and a faint lower band that was similar in molecular weight (Figure 4A). An extended exposure time was required to detect wild-type EGFR by Western blot and, as a result, the lower band of the EGFRvIII doublet was poorly defined due to the strong intensity of the upper band. However, the definition between the lower and upper band for EGFRvIII is more apparent in later figures (see Figures 5 and 6). By carefully aligning blots probed with different antibodies, I found that the molecular weight of the Flag-detectable product was equal to that of the lower band of the total-EGFR doublet at approximately 140KDa, in agreement with our previous work (Lavictoire et al, 2003). The specific phospho-Y1068 antibody and the general phosphotyrosine antibody, PY-20, each showed only a single phosphorylated species of EGFRvIII at the molecular weight of the upper band corresponding to mature EGFRvIII. The second, lower band in the PY-20 blot was found in both cell lines, but it was not detected with the phospho-Y1068 antibody, which suggests that the band represents an approximately 115KDa tyrosine-phosphorylated protein that is not EGFR or EGFRvIII. From this experiment I concluded that no phosphorylation of nascent EGFRvIII is noticable by Western blot of total cell lysates.

Interestingly, wild-type EGFR protein was absent from U87tftΔEGFR cells, which are identical to U87MG cells, except that they have been transduced and selected to express Flag-tagged EGFRvIII and the Moloney murine leukemia virus ecotropic receptor. We are unsure why wild-type EGFR protein expression was absent from these cells, however we did

Figure 4: Phosphorylation state of nascent EGFRvIII.
A) Western blotting was used to characterize the nascent and mature forms of Flag-tagged EGFRvIII using anti-Flag, -EGFR, and -phosphotyrosine antibodies. Total cell lysates from U87MGtftΔEGFR cells, which expressed a triple Flag-tagged EGFRvIII, were subjected to Western blot analysis. Lysates from U87MG cells, which express EGFR, were used for relative comparison. Western blots were aligned based upon molecular weight markers to show differences in weight between the forms of EGFRvIII as detected using EGFR Ab-12, and an anti-Flag antibody. The phospho-tyrosine antibody PY-20, has a general affinity to phosphorylated tyrosine residues, meaning it may recognize other proteins besides EGFR and EGFRvIII. The antibody to phospho-Y1068 specifically recognizes one of the 5 autophosphorylation sites on EGFR and EGFRvIII. B) Phosphorylation of nascent EGFRvIII was detected by immunoprecipitation, followed by Western blot. Nascent EGFRvIII was selectively purified using anti-Flag resin, and the purified fractions were separated by SDS-PAGE and analyzed by Western blot for Flag and phospho-Y1068. Lysate from U87MGtftΔEGFR cells, which express un-tagged EGFRvIII, were used as a negative control Data is representative of at least 2 independent experiments.


A
U87MG
U87tftDeltaEGFR
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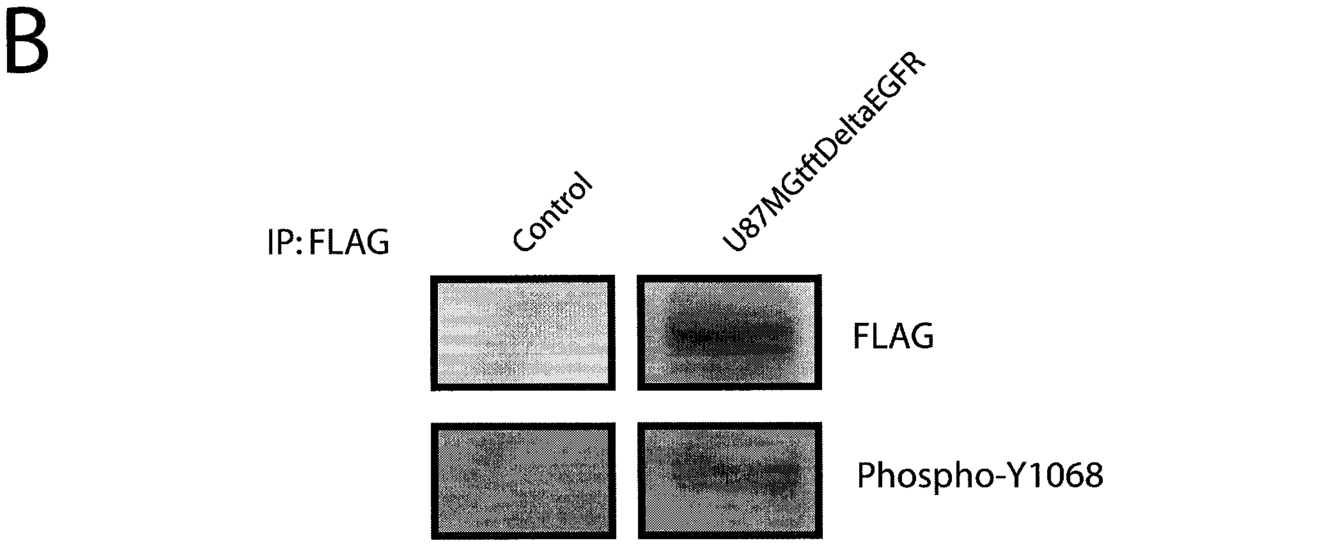
U87MGtftDeltaEGFR
U87MG
U87MGtftDeltaEGFR
U87MG
U87MGtftDeltaEGFR
170KDa
130KDa
EGFR
FLAG
Phospho-Y1068
PY-20


B
IP: FLAG
Control
U87MGtftDeltaEGFR
FLAG
Phospho-Y1068

not feel that it would impact our experiments studying the biogenesis of EGFRvIII as the EGFR protein was consistently absent in all experiments.

Since no phosphorylation of nascent EGFRvIII was observed by Western blot of total cell lysates, I decided to use a more sensitive approach using immunopurified nascent receptor. I immunoprecipitated nascent EGFRvIII from U87tftΔEGFR cells using the Flag-epitope and looked for the presence of tyrosine phosphorylation by Western blot (Figure 4B). By this technique, I detected a single band using the phospho-Y1068 antibody at the molecular weight of the Flag-tagged nascent receptor, indicating some tyrosine phosphorylation on nascent EGFRvIII. These data suggest that EGFRvIII may dimerize to a small extent within the ER, however the proportion of active, nascent EGFRvIII is likely very small compared to that of mature receptor since it was not observable by Western blot of total cell lysates. Having shown that Flag-detectable EGFRvIII is an immature form of the protein with a limited capacity for activation, I then sought to confirm its designation as an Hsp90 client protein.

3.2 Nascent EGFRvIII is a client protein of Hsp90

Many proteins have been identified as clients of Hsp90. Client proteins depend upon Hsp90 for stability, and/or activity. Loss of Hsp90 function in these instances will lead to degradation of the misfolded protein, or loss of function. Although requirement for Hsp90 varies, defining new client proteins involves two major defining characteristics. First, a client protein must have a physical interaction with Hsp90. In some instances, the requirement for Hsp90 may be minimal or transient, but this interaction must be observed. Secondly, the client protein must require Hsp90 activity to remain stable, active, or

activation-competent, depending on its specific situation. This second step is important as it differentiates client proteins from cochaperones and other non-client interactors, which also fulfill the first requirement.

To confirm a dependence of tftΔEGFR upon Hsp90 activity, U87tftΔEGFR were treated with geldanamycin (GA) or a vehicle control. Concentrations of GA were chosen to selectively inhibit Hsp90, and not its ER homologue, Grp94, which has a reduced affinity for the drug (Xu et al, 2001). Treatment with GA produced a significant reduction in protein levels of both nascent (Flag-detectable) and mature EGFRvIII, as well as loss of tyrosine phosphorylation. Meanwhile, no change was observed on total Erk protein levels, which do not rely on Hsp90 for protein stability (Figure 5). The physical interaction between the two proteins was proven by co-immunoprecipitation as described later in this report (Figure 8).

Having confirmed that the nascent form of EGFRvIII is a client of Hsp90, I then focused on examining the role of Cdc37 in EGFR stability.

3.3 Effect of Cdc37 Overexpression on EGFRvIII

To examine the importance of Cdc37 in the biogenesis of EGFRvIII, U87 glioblastoma cells expressing EGFRvIII were transduced with a retrovirus coding for wild-type Cdc37 (Cdc37 WT). Infected cells were selected using puromycin to ensure the population of surviving cells overexpressed Cdc37 WT. Western blot analysis of these Cdc37 WT transduced cells compared to cells transduced with a control vector showed a strong overexpression of Cdc37, which was associated with an increase in both the upper and lower (mature and immature, respectively) bands of EGFRvIII (Figure 6A, lanes 1 and 4). This showed that there is a positive role for Cdc37 in EGFRvIII expression. I then chose

Figure 5: Effect of geldanamycin on EGFRvIII.
Client proteins of Hsp90 are often degraded following Hsp90 inhibition. To confirm the effects of the Hsp90 inhibitor Geldanamycin upon EGFRvIII, U87MGtftΔEGFR cells were plated at $2x10^6$ cells per well in a 6 well dish and treated with 300nM or 500nM geldanamycin or equivalent DMSO control as outline in "Materials and Methods". Lysates were taken 16 hours after treatment and proteins levels were analyzed by Western blot. 80μg of protein were loaded in each lane. Data shown are representative of two independent experiments.

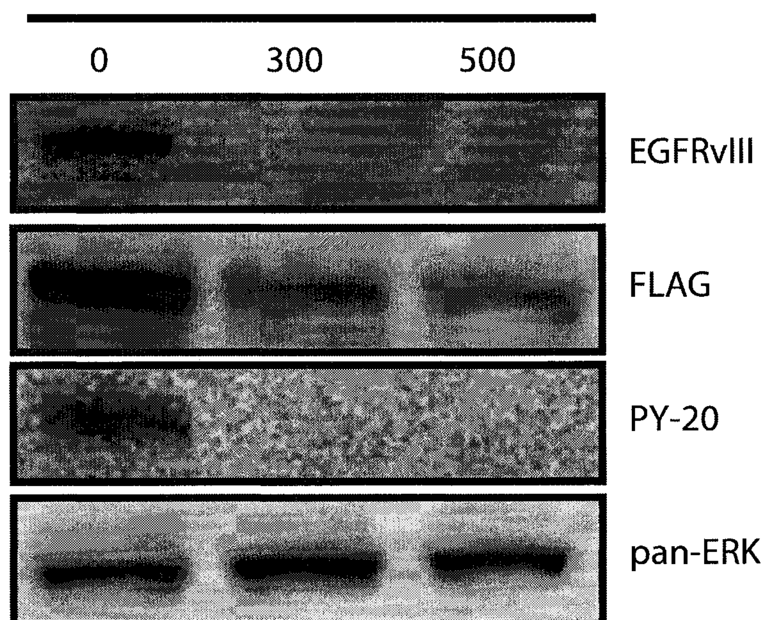
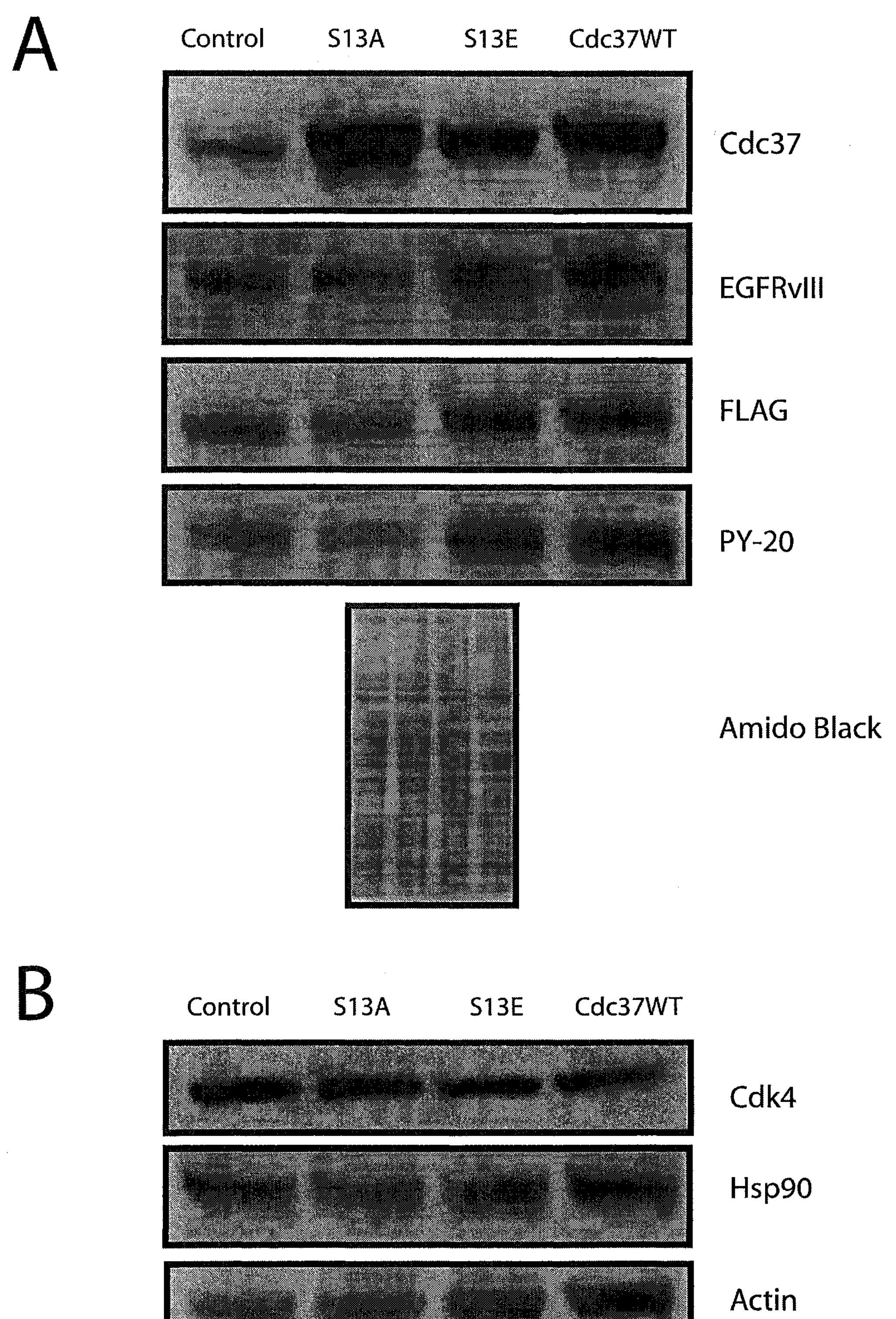

Geldanamycin (nM)
0
300
500
EGFRvIII
FLAG
PY-20
pan-ERK

Figure 6: Effects of Cdc37 wild-type and mutant expression on EGFRvIII, Cdk4, and Hsp90.

Cdc37 WT, Cdc37 S13A, and Cdc37 S13E were stably expressed in U87MGtftΔEGFR cells using a retroviral vector. Infected cells were selected using puromycin for at least one week. Total cell lysates were collected, separated by SDS-PAGE and protein levels of A) Cdc37, EGFRvIII, B) Cdk4, and Hsp90 were analyzed by Western blot. Control lane represents cells transduced with an empty vector control virus. 80μg of protein was loaded in each lane. Data are representative of at least 2 independent experiments. Pan-Erk was probed as a loading control.


A
Control
S13A
S13E
Cdc37WT
Cdc37
EGFRvIII
FLAG
PY-20
Amido Black
B
Control
S13A
S13E
Cdc37WT
Cdk4
Hsp90
Actin

to examine the nature of the Cdc37/EGFRvIII relationship more closely using Cdc37 serine 13 mutants and Flag-tagged EGFR.

3.4 Construction and Expression of Cdc37 Mutants

Mutations of serine 13 on Cdc37 have been shown to alter its ability to bind certain client proteins, and their recruitment to Hsp90. Having shown a positive role for Cdc37 in EGFRvIII expression, the goal of this experiment was to determine if stably overexpressing Cdc37 mutants would alter the stability of nascent and mature EGFRvIII in the U87tftΔEGFR glioblastoma cell line. Two Cdc37 mutant constructs, S13A and S13E were created by site directed mutagenesis to convert serine 13 to alanine and glutamic acid, respectively.

Using a three-plasmid transfection system, ecotropic, non-replicating retroviruses were created containing Cdc37 WT, S13A, S13E, or control vector (Soneoka et al, 1995). These viruses were used to transduce U87tftΔEGFR cells, which express an ecotropic receptor. Following transduction, infected cells were selected for puromycin resistance, and maintained for experimental use. Transduction of these vectors yielded expression of Cdc37 protein at respective levels of approximately 500, 280, and 350% for Cdc37 S13A, S13E and WT over the endogenous Cdc37 in Control transduced cells (Figure 6A).

3.5 Effect of Cdc37 Serine 13 Mutants on EGFRvIII, Hsp90, and Cdk4 Protein Levels.

Having stably transduced cells to express Cdc37 WT, Cdc37 S13A, or Cdc37 S13E, I then studied their effects on EGFRvIII biogenesis. Relative to control transduced cells, overexpression of a Cdc37 WT protein resulted in a 50% increase in levels of total, mature EGFRvIII, and a 40% increase in nascent EGFRvIII, as detected with the anti-Flag antibody (Figure 6A). This increased pool of mature EGFRvIII also showed an enhanced phosphotyrosine signal, used as a marker of its activity. Meanwhile, expression of Cdc37 S13A protein, which was designed to function as an inactive, non-phosphorylatable Cdc37, resulted in no change on nascent and mature EGFRvIII protein, as well as a slightly diminished phosphotyrosine activity. Cdc37 S13E protein expression, which was expected to partially mimic active, phosphorylated Cdc37, expressed at a slightly lower level than wild-type Cdc37, but yielded identical increases in EGFRvIII protein levels and level of activity. This indicated the importance of serine 13 on Cdc37 and suggested a positive role for its phosphorylation during EGFRvIII biogenesis.

I also examined the affects of Cdc37 mutant expression upon the levels of Hsp90, and the Cdc37/Hsp90 client protein, Cdk4. As a client of Cdc37 and Hsp90, I expected to see increases in Cdk4 with expression of Cdc37 WT and S13E. Instead, Cdk4 levels did not increase, and decreased by 10% relative to control cells (Figure 6B). Cdk4 levels in Cdc37 S13A cells were identical to the control. Hsp90 levels were fairly even between the cell lines, with the slightly higher levels seen in Cdc37 WT cells being accounted for by an increase in the actin loading control.

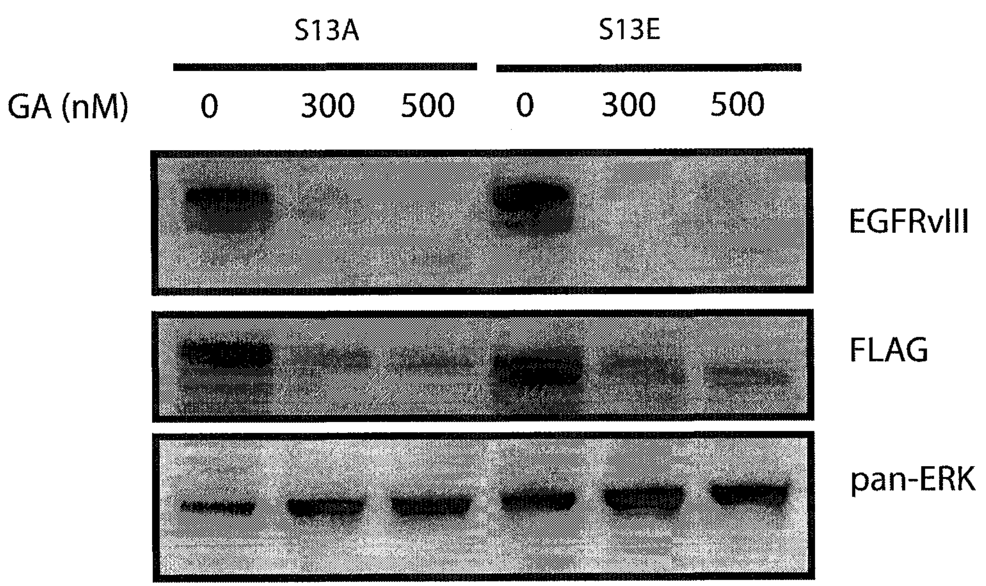
3.6 Cdc37 Mutants Function in an Hsp90-dependent Manner

Cdc37 has previously been shown to chaperone the folding of a small number of client proteins independently of Hsp90 (Rao et at, 2001; Kimura et al, 1997). To determine whether the enhanced levels of EGFRvIII upon Cdc37 S13E mutant expression are dependent upon Hsp90, I treated these cells with the Hsp90 inhibitor, geldanamycin (GA, Figure 7). Although the Cdc37 S13A mutant did not affect EGFRvIII levels, I treated those cells with GA, as well, to ensure that it did not effect the sensitivity of the Hsp90 system to such inhibitors. In both cases, EGFRvIII proved to be sensitive to Hsp90 inhibition, with the mature protein being undetectable by Western blot at 300nM treatment. The similarities in total and nascent EGFRvIII levels in untreated lanes between the two cell lines was the result of extended exposure of the Western blot to maximize the signal from treated samples. Having shown that these Cdc37 mutants work in cooperation with Hsp90, I then analyzed their abilities to bind and recruit Hsp90 to the nascent protein.

3.7 Binding of Cdc37 Mutants to Nascent EGFRvIII

Previous studies have shown that a S13A mutation on Cdc37 limits Cdc37 and Hsp90's ability to bind client proteins (Miyata and Nishida, 2004). These data suggested an important role for phosphorylation of serine 13 in client recruitment to the Cdc37/Hsp90 complex. Only one study has looked at the binding of Cdc37 S13E, showing it had stronger binding than S13A, but weaker than wild-type Cdc37 that was largely in its phosphorylated form (Shao et al, 2003). To explore this with respect to EGFRvIII, I immunoprepitated the nascent EGFRvIII protein using the triple Flag-epitope from control, Cdc37 WT, S13A and S13E transduced cell lines. U87ΔEGFR cells, which express untagged EGFRvIII, were used

Figure 7: Cdc37 mutants function in an Hsp90-dependent manner.
To confirm that Cdc37 S13A and S13E mutant proteins do not have Hsp90-independent activities on EGFRvIII biogenesis, cells were treated with the Hsp90 inhibitor geldanamycin. U87MGtftΔEGFR/ Cdc37S13A, and Cdc3713E cells were plated at $2x10^6$ cells per well in a 6 well dish and treated with 60nM or 120nM Geldanamycin or equivalent DMSO control as outline in "Materials and Methods". Lysates were taken 16 hours after treatment and proteins levels were analyzed by Western blot. 80μg of protein were loaded in each lane. Data shown are representative of two independent experiments. Erk protein levels are used as a loading control.

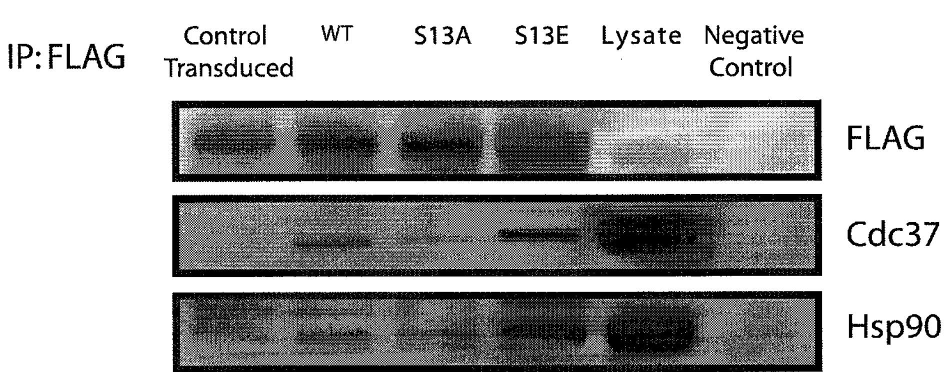

S13A
S13E
GA (nM)
0
300
500
0
300
500
EGFRvIII
FLAG
pan-ERK

as a negative control. Immunopurified nascent EGFRvIII complex was separated by SDS-PAGE and bound proteins were analyzed by Western Blot (Figure 8). Levels of Cdc37 were quantified for relative comparison. The highest level of binding of Cdc37 to nascent EGFRvIII was seen with Cdc37 S13E. Approximately 40% less Cdc37 WT immunoprecipitated with nascent EGFRvIII compared to Cdc37 S13E. Although the amount of nascent EGFRvIII pulled down from the Cdc37 S13E cells appears slightly higher than the other lanes, the increased association of Cdc37 and Hsp90 was consistently observed between experiments. Control transduced cells showed an undetectable amount of associated Cdc37, while Cdc37 bound to nascent EGFRvIII in Cdc37 S13A cells was just barely detectable at 1% that of Cdc37 S13E. For the most part, Hsp90 binding appeared to correlate with Cdc37 binding, with the greatest association between nascent EGFRvIII and Hsp90 seen in S13E cells, and the lowest in the control transduced cells. The levels of bound Hsp90 in Cdc37 WT and Cdc37 S13A cells were similar and approximately 90% less than levels in Cdc37 S13E cells. A low amount of background Hsp90 binding was also detected in the negative control, representing a background level of binding to the antibody or incomplete washing of this abundant protein. This experiment showed the importance of serine 13 on Cdc37 for its association with nascent EGFRvIII, and indicated that Hsp90 binding may be dependent upon the association of Cdc37 to the client.

3.8 Examination of Cdc37 Phosphorylation Status

In order to properly compare the effects of Cdc37 WT expression with Cdc37 S13E, which was intended to function as a phospho-mimetic, I looked to determine the phosphorylation status of the population of overexpressed Cdc37 WT. Phosphorylation of

Figure 8: Association of Cdc37 and Hsp90 with nascent EGFRvIII.
To examine the importance of serine 13 of Cdc37 in its ability to recruit Hsp90 to nascent EGFRvIII, nascent EGFRvIII was selectively purified using its Flag epitope and associated proteins were analyzed by Western blotting following SDS-PAGE. U87MGtftΔEGFR / Control, Cdc37 WT, S13A, and S13E transduced cells were plated at $4x10^6$ cells per 10cm plate and harvested for immunoprecipitation after 24 hours. Untagged U87MGDeltaEGFR lysate was used as a specificity control and total cell lysate from U87MGtftDeltaEGFR/Cdc37 S13E cells was used as a positive Western blot control. Nascent EGFRvIII was selectively purified using anti-Flag resin, and the purified fractions were separated by SDS-PAGE and analyzed by Western blot. Data are representative of at least 2 independent experiments.


IP: FLAG
Control Transduced
WT
S13A
S13E
Lysate
Negative Control
FLAG
Cdc37
Hsp90

serine 13 is the only modification known to occur with Cdc37. Previous studies using mass spectrometry have identified two natural forms of Cdc37: phosphorylated and non-phosphorylated (Shao et al, 2003). I chose to use 2D gel electrophoresis to separate these intracellular forms of Cdc37. As a control, I examined Cdc37 S13A, which cannot be phosphorylated. As expected, the control sample showed only one form of Cdc37, which, based on previous reports, represents the non-phosphorylated form of Cdc37 (Figure 9). Examining Cdc37 WT, I discovered two equally abundant species, one form at the same isoelectric point as in the S13A mutant, and another of more acidic isoelectric point. Since only two forms of Cdc37 have been found to exist, this second form is most likely the phosphorylated Cdc37. Combination of equal parts of Cdc37 S13A and Cdc37 WT lysate showed overlap with one form of Cdc37 WT, suggesting that the other species is the phosphorylated form. Based on the results of previous studies (Shao et al, 2003), I concluded that the Cdc37 population in the Cdc37 WT expressing cells is approximately 50% phosphorylated on serine 13.

3.9 Effect of Cdc37 on the Cell Cycle

Cdc37 is known as a regulator of the cell cycle in yeast (Gerber et al, 1995), and has been shown to have a role in promoting proliferation in mammalian tumours (Pascale et al, 2005). To analyze the effects of stable Cdc37 WT, S13A and S13E expression on the cell cycle, I used propidium iodide staining on sub-confluent cells followed by flow cytometry. To ensure that the growth factors present in the serum in the culture media did not mask the effects of increased EGFRvIII activity, I looked at cells under both normal (10%) and low (0.5%) serum conditions (Figure 10). Special care was taken to ensure that

Figure 9: Examination of Cdc37 phosphorylation by two-dimensional gel electrophoresis.

The degree of Cdc37 in the U87MGtftΔEGFR/Cdc37 WT cell lines phosphorylation was determined by 2D-gel electrophoresis. Briefly, cell lysates were extracted, and separated by isoelectric focusing on a pH 5-7 IPTG strip. Proteins from the strip were then separated in a second dimension by SDS-PAGE, and Cdc37 was detected by Western blotting. Lysate from U87MGtftΔEGFR/Cdc37 S13A cells was used as a non-phosphorylated Cdc37 control. In the Cdc37 WT + S13A experiment, equal parts lysate were combined from both cell lines to identify which species represents the non-phosphorylated form of Cdc37.

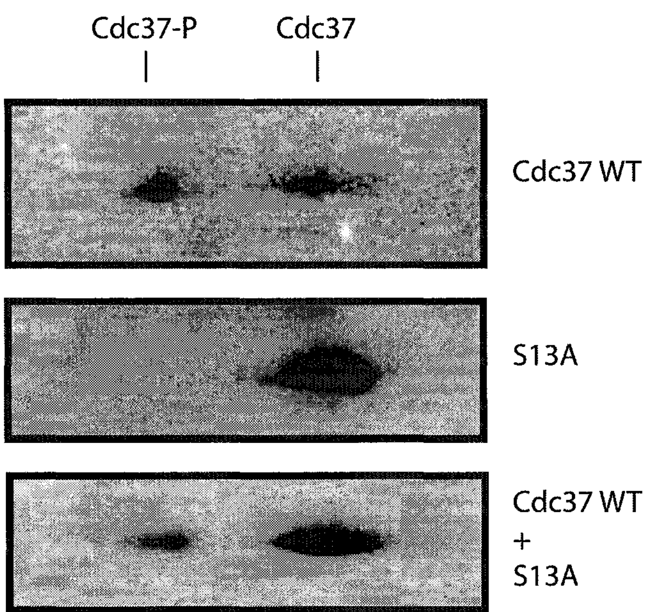

Cdc37-P
Cdc37
Cdc37 WT
S13A
Cdc37 WT
+
S13A

Figure 10: Effects of Cdc37 mutant expression on cell cycle.
To observe the effect of Cdc37 overexpression and expression of Cdc37 S13A and S13E on the cell cycle, I performed cell cycle analysis by flow cytometry using propidium iodide staining. Cells were plated at low density in either A) 10% Serum, or B) 0.5% serum conditioned media, then collected after 24 hours growth, fixed in ethanol, then stained with propidium iodide containing RNase A. Data are representative of 3 independent experiments, performed in triplicate and are presented in table and graphical format. Standard deviation of the mean is shown for all data. On graphs, G1 phase is the black bar, G2/M is shown as white, and S phase is grey. Data were collected on a Beckman-Coulter Epics XL flow cytometer, and analyzed using ModFit LT software. Gates were designed to exclude only aggregated cells. C) Representative flow cytometric analyses of control cells grown under 0.5% serum. Red areas are calculated G1 (left) and G2/M (right) cell populations as determined by ModFit LT. The enclosed area between red peaks shows the S phase population.

A

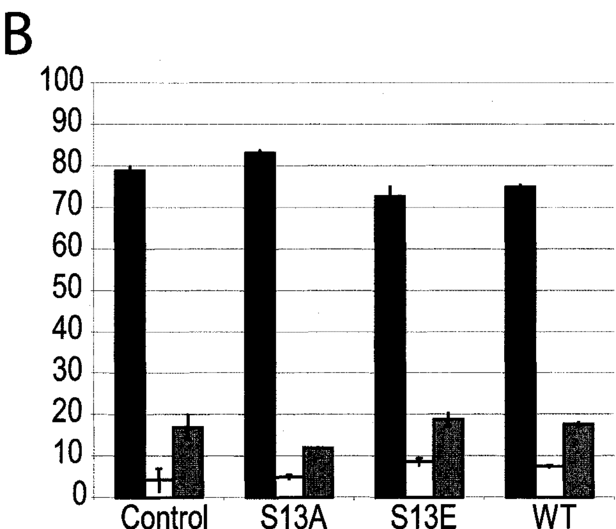
0 10 20 30 40 50 60 70 80 90 100

Control S13A S13E WT

Cell cycle population percentages under 10% serum as determined by flow cytometry

Cell line	G1	G2/M	S
	Cell Cycle Phase (%)		
Control	60.1 ±3.1	12.9 ±1.8	26.3 ±1.4
Cdc37 S13A	60.2 ±2.2	11.9 ±1.2	27.9 ±1.2
Cdc37 S13E	62.0 ±1.8	12.2 ±1.9	25.8 ±1.2
Cdc37 WT	57.2 ±2.6	14.6 ±1.6	28.3 ±1.2

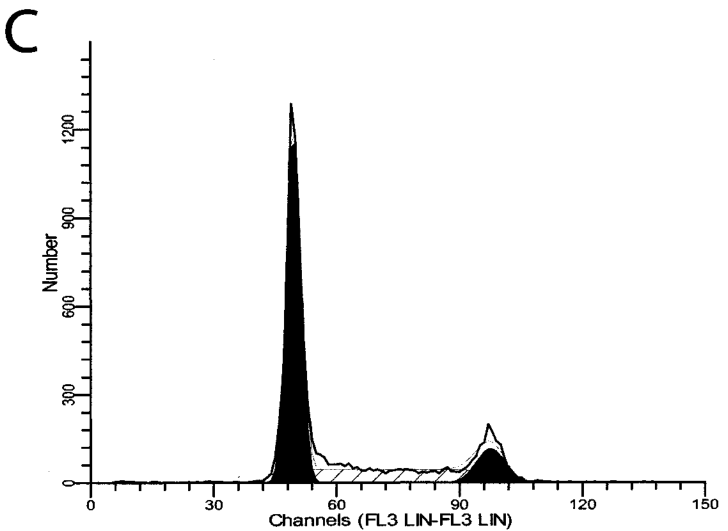
B



Cell cycle population percentages under 0.5% serum as determined by flow cytometry

Cell line	G1	G2/M	S
	Cell Cycle Phase (%)		
Control	78.8 ±1.0	4.2 ±2.7	17.0 ±2.9
Cdc37 S13A	83.1 ±0.5	4.9 ±0.5	12.0 ±0.1
Cdc37 S13E	72.6 ±2.4	8.6 ±0.9	18.8 ±1.5
Cdc37 WT	74.9 ±0.5	7.5 ±0.2	17.7 ±0.4

C

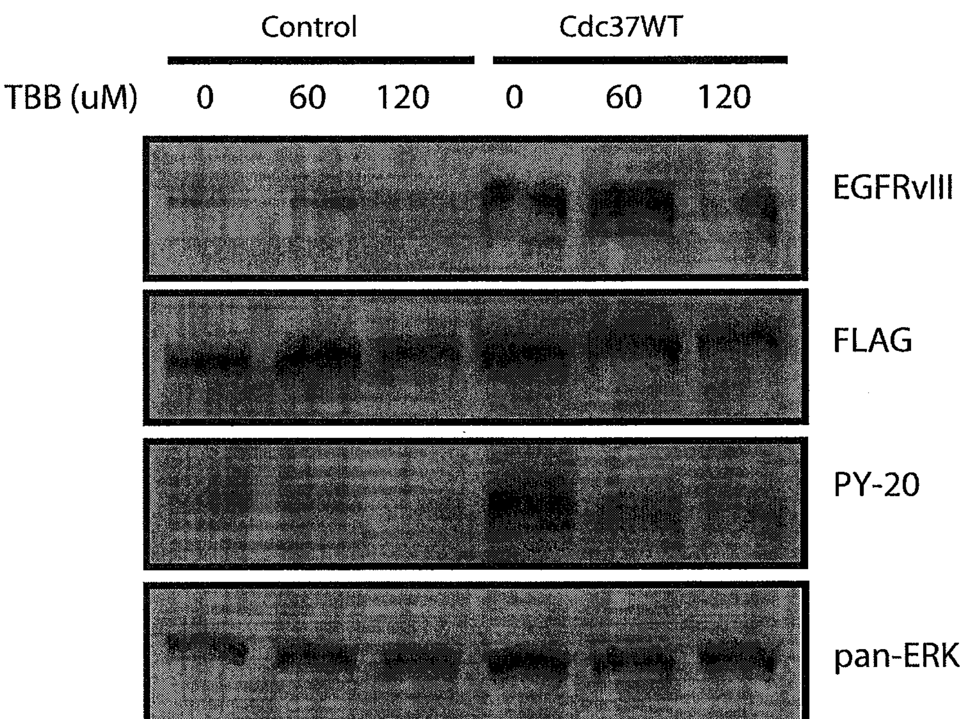


cells were plated at densities well below confluence to minimize growth inhibition caused by high cell density. A two-tailed independent T-test was used to determine statistical significance of cell cycle differences. No differences were seen between the five cell lines when grown in 10% serum (10A). Low serum conditions resulted in increased numbers of cells in the G1 phase, and lower S and G2/M populations compared to 10% serum. Under 0.5% serum conditions (10B), Cdc37 WT and S13E cells had a small (~5%), but statistically significant decreased population of cells in the G1 phase than control cells ($P < 0.01$ and 0.02). Small increases in S and G2/M populations were seen, but these were not statistically significant. Taken together, these data showed that Cdc37 overexpression does not cause any major changes on the cell cycle of U87tftΔEGFR cells, but a small, negative effect is seen on the G1 phase population in Cdc37 WT and S13E cells when the effects of exogenous growth factors are reduced in low serum conditions.

3.10 Effect of CK2 Inhibition on EGFRvIII

I next tested to see if inhibitors could be used to decrease EGFRvIII biogenesis by preventing CK2-mediated phosphorylation of Cdc37. For this, I used the CK2 inhibitor TBB, which has previously been shown to reduce the amount of phosphorylated Cdc37 in cell culture and lower the amount of Cdc37 bound to client proteins (Nishida and Miyata, 2004). For this experiment, I treated control and Cdc37 WT transduced U87tftΔEGFR cells with TBB, then examined the effects by Western blot (Figure 11). Vehicle-only treatments were used as controls. In both cell lines, a decrease in the amount of total EGFRvIII was seen with treatment with 120 μM TBB, but not at 60 μM. Both concentrations, however, greatly reduced the tyrosine phosphorylation on EGFRvIII, which is used to indicate the

Figure 11: Effects of TBB treatment on EGFRvIII.
CK2 is the only kinase known to phosphorylate Cdc37. The effects of CK2 inhibition on nascent and mature EGFRvIII were determined using the CK2 inhibitor TBB. U87MGtftΔEGFR/Cdc37 WT and Control cells were plated at $2x10^6$ cells per well in a 6 well dish and treated with TBB or DMSO control. Lysates were taken 16 hours after treatment, separated by SDS-PAGE and protein levels were analyzed by Western blot. 80μg of protein were loaded in each lane. Data are representative of 2 independent experiments.

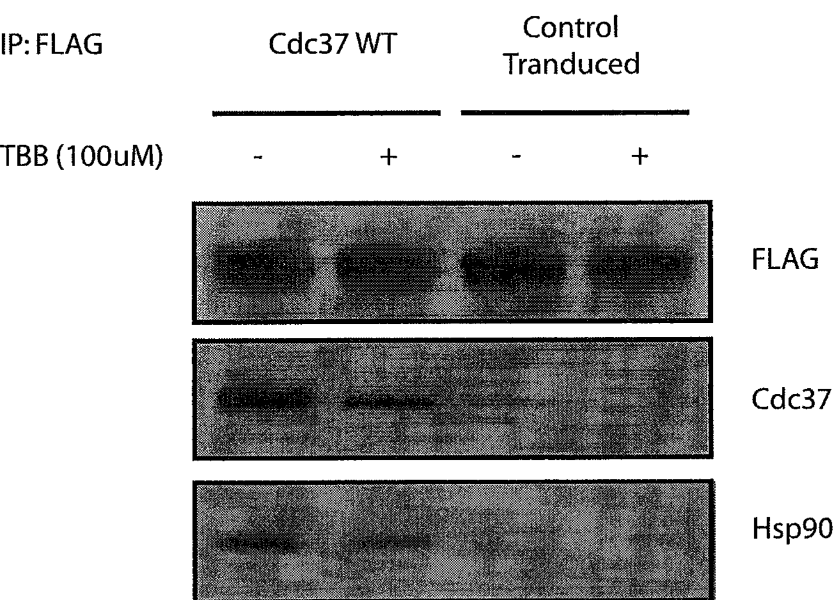

Control
Cdc37WT
TBB (uM)
0
60
120
0
60
120
EGFRvIII
FLAG
PY-20
pan-ERK

status of its activation. Levels of nascent EGFRvIII, detected using an anti-Flag antibody, were unchanged with 60 μM TBB treatment, while being slightly decreased with the 120 μM concentration. This experiment outlined an important role for CK2 activity in the expression and activity of mature EGFRvIII. It also showed a limited effect of CK2 inhibition on the protein levels of nascent EGFRvIII.

3.11 Role of CK2 in Regulating EGFRvIII Recruitment to the Cdc37/Hsp90 Complex

Having found that CK2 inhibition slightly reduces the levels of nascent and mature EGFRvIII, I next examined the effect this treatment had on the association of nascent EGFRvIII to the Cdc37/Hsp90 complex. I treated Control and Cdc37 WT transduced cells with 120 μM TBB and used the Flag epitope to selectively immunoprecipitate nascent EGFRvIII from these cells (Figure 12). Vehicle-only treatments were used as controls. Purified proteins were separated by SDS-PAGE and analyzed by Western blotting. Treatment of Cdc37 WT cells with TBB reduced the amount of Cdc37 bound to nascent EGFRvIII by 70%. Approximately 60% less Hsp90 was also bound to nascent EGFRvIII after treatment. In the control transduced cells, no effect was seen on the already low level of Cdc37 binding following treatment but a slight reduction of Hsp90 binding was observed. These data showed for the first time that CK2 inhibition limits the association of Cdc37 and Hsp90 with a client protein, and reveals CK2 as a target to limit chaperone binding to nascent EGFRvIII.

Figure 12: Association of Cdc37 and Hsp90 with nascent EGFRvIII following TBB treatment.
CK2 inhibition is known to reduce Cdc37 phosphorylation. To see if CK2 activity is necessary for the binding of Cdc37 and Hsp90 to nascent EGFRvIII, the nascent receptor was immunoprecipitated from cells following 16 hours of CK2 inhibition and bound Hsp90 and Cdc37 were examined. U87MGtftΔEGFR/ Control and Cdc37 WT cells were plated at $2x10^6$ and treated with 120μM TBB or DMSO control 24 hours after seeding. Following 16 hours of treatment, lysates were collected for immunoprecipitation. Nascent EGFRvIII was selectively purified using anti-Flag resin, and the purified fractions were separated by SDS-PAGE and analyzed by Western blot. Data are representative of 2 independent experiments.



IP: FLAG
Cdc37 WT
Control
Tranduced
TBB (100uM)
-
+
-
+
FLAG
Cdc37
Hsp90

Chapter 4: Discussion

We have previously identified nascent EGFRvIII as a client of the Cdc37/Hsp90 chaperone complex (Lavictoire et al, 2003). Cdc37's role is to recruit certain kinase proteins to Hsp90 for folding and its ability to bind these kinases is thought to be dependent upon phosphorylation of serine 13 in its client-binding domain, however this has never been studied for a protein which is synthesized on the ER. I hypothesized that Cdc37 may control EGFRvIII biogenesis by regulating its recruitment to Hsp90. Since phosphorylation of Cdc37 is thought to be necessary for its recruitment of many client proteins, it may also be a means by which to control this process using targeted drugs. I sought to determine three things: the role of Cdc37 in EGFRvIII biogenesis, the importance of Cdc37 phosphorylation in its interaction with EGFRvIII, and the effect of CK2 inhibition on EGFRvIII stability and chaperone recruitment. To study this, I used a U87tftΔEGFR cell line that expresses an EGFRvIII protein with a triple-Flag epitope which is only detectable on the protein in the endoplasmic reticulum, and can, therefore, be used to selectively study EGFRvIII in its nascent form. Cells were transduced to stably express wild-type and mutant Cdc37 proteins. The Cdc37 mutants were designed based upon previous studies to create a non-phosphorylatable Cdc37 (Cdc37 S13A), and a mimic of phosphorylated Cdc37 (Cdc37 S13E) (Shao et al, 2003).

With respect to the first objective, I found that overexpression of Cdc37 WT increased levels of EGFRvIII protein in both its nascent and mature form (Figure 6). The increase of nascent EGFRvIII not only indicates an involvement of Cdc37 in EGFRvIII biogenesis, but also suggests that endogenous levels of Cdc37 are normally a limiting factor in this process. Increased EGFRvIII levels following Cdc37 overexpression were associated

with increased Cdc37 and Hsp90 recruitment (Figure 8), suggesting that Cdc37's role in EGFRvIII biogenesis is related to its ability to recruit Hsp90 to the client. This means that Cdc37 plays a postive role in EGFRvIII biogenesis and reducing the amount of available, active Cdc37 in a tumour could have potential therapeutic uses.

The fact that Cdc37 overexpression also increases the amount of both nascent and mature EGFRvIII protein within the cell suggests that a significant amount of nascent EGFRvIII, which has the potential to reach its mature form, does not normally mature due to a lack of chaperone assistance. Thus, increasing the recruitment of nascent EGFRvIII to Cdc37 and Hsp90 may promote its maturation and protect it from degradation. Proteins that misfold in the ER are degraded by a process known as ER-associated degradation (ERAD; reviewed in Romisch, 2005). This quality-control process involves the retrograde transport of a misfolded protein from the ER to the cytosol for degradation mediated by the proteasome. While degradation of misfolded nascent proteins is understood to protect cells from stress (Oyadomari et al, 2006), the proteins involved in designating a protein for ERAD, as well as the method of dislocation of the protein through the ER membrane are ill defined. Here, I have shown for the first time that Cdc37 acts upon a protein in the ER to promote its maturation. This suggests a novel role for Cdc37 in antagonizing ERAD-mediated degradation of some proteins.

The second objective of the study was to determine the importance of Cdc37 serine 13 phosphorylation in Cdc37's interaction with nascent EGFRvIII. Expression of the Cdc37 S13A mutant, which lacks the ability to become phosphorylated, had little effect on EGFRvIII expression or the binding of Cdc37 and Hsp90 to nascent EGFRvIII (Figures 6 and 8). This finding concurred with a previous report that the same mutant had a severely

impaired ability to bind Hsp90 or client (Shao et al, 2003) and fits a model where unphosphorylated Cdc37 acts as a bystander rather than a competitor because it does not interact with client or Hsp90, and, thus, does not interfere with the function of active, phosphorylated Cdc37.

The Cdc37 S13E mutant was designed to act as a mimic of phosphorylated Cdc37. Cells expressing Cdc37 S13E showed levels of nascent and mature EGFRvIII equal to those seen when Cdc37 WT was overexpressed. Interestingly, three times more Cdc37 immunoprecipitated with nascent EGFRvIII in the Cdc37 S13E than Cdc37 WT cells (Figure 8). These results were surprising considering a previous report which found this mutant had only 15% the affinity for client as phosphorylated wild-type Cdc37 (Shao et al, 2003). One possible explanation for this discrepancy stems from many inherent differences between the studies. Namely, I looked at nascent EGFRvIII within the ER membrane in glioblastoma cell lines that stably express Cdc37 constructs, while the previous study looked at mature HRI within the cytosol of rabbit reticulocyte lysates. Since the dependence of client proteins for Cdc37 and Hsp90 varies between proteins, and because there appears to be no conserved motif among clients for Cdc37 binding, the particulars of this interaction may be somewhat unique in each case.

An alternative interpretation of the function of the Cdc37 S13E mutant stemmed from my analysis of the phosphorylation state of the Cdc37 WT protein. It was important to determine the percent phosphorylation of Cdc37 WT in order to properly compare its activity to the mutant and determine its importance in EGFRvIII biogenesis. Since no antibodies are available to specifically identify phosphorylated Cdc37, I used two-dimensional gel electrophoresis to separate the different forms. Previous studies using mass

spectrometry have identified two natural forms of Cdc37: phosphorylated and non-phosphorylated (Shao et al, 2003). Using two-dimensional gels, I identified two equally abundant species of Cdc37 WT (Figure 9). Cdc37 S13A lysate, which cannot become phosphorylated on serine 13, showed only a single species, which overlapped with the more basic of that with Cdc37 WT when lysates were combined in equal quantities. Although, issues with consistently resolving the two protein species in Cdc37 WT cells restricted further study, I presumed that the second identified species of Cdc37 WT was the phosphorylated form because phosphorylation of serine 13 is the only modification known to occur to Cdc37, and mutation of this site eliminated one species on 2D gel. On this assumption, since both species of Cdc37 in Cdc37 WT cells were equivalent in size, this meant that the population of Cdc37 WT was 50% phosphorylated. Therefore, these results suggest that a less active Cdc37 WT protein was able to produce the same effect on the EGFRvIII biogenesis as Cdc37 S13E. Therefore, the Cdc37 S13E mutant may have acted as only a partial mimic of phosphorylated Cdc37 since more of it was needed to produce to same result. On the other hand, a surplus of Cdc37 activity may have allowed another factor to become limiting in the biogenesis of EGFRvIII, causing its levels to plateau. In terms of my objective, the data from the serine 13 mutants are consistent with an essential role for serine 13 phosphorylation of Cdc37 on EGFRvIII biogenesis.

The third objective of this study involved using CK2 inhibitors to reduce the presence of phosphorylated Cdc37 within a cell. It has been shown that treatment of cells with the CK2 inhibitor TBB greatly reduces the amount of phosphorylated Cdc37 (Miyata and Nishida, 2005) and reduces the total protein levels of several Cdc37/Hsp90 clients. Similarly, I found that TBB treatment decreased protein levels of both nascent and mature

EGFRvIII (Figure 11), and that this decrease was associated with a reduced binding of Cdc37 and Hsp90 to the client (Figure 12), which has never been examined for any client protein. One possible reason that the change in Cdc37 binding after treatment was not obvious within control cells is because the levels of chaperone and cochaperone binding are already at the detection limits for the assay. For this reason, I also used Cdc37 WT cells for the experiment because they had shown a higher association between Cdc37 and EGFRvIII (Figure 8), and any change in Cdc37 binding would be more evident, as was the case. It is interesting to note that for Cdc37 WT cells, a decrease in the tyrosine phosphorylation of the mature protein was observed with 60μM TBB treatment, which did not cause reduction of EGFRvIII protein levels (Figure 11). This suggests that the mature EGFRvIII, which is normally constitutively active, was somehow unable to activate following CK2 inhibition and may indicate loss of conformation due to disruption of the chaperone system. It should be made clear, however, that these experiments do not rule out possibility of chaperone-independent effects resulting from CK2 inhibition.

Another noteworthy observation in the CK2 inhibition experiment was the relatively small decrease in nascent EGFRvIII protein compared to the relatively large decrease in its association with Cdc37 and Hsp90 (Figure 11). I predicted that the reduced association with the Cdc37/Hsp90 complex might cause a more dramatic change on nascent protein levels. It is likely that other factors that control protein turnover, such as translation and degradation rates could account for such a finding. In fact, following geldanamycin treatments, the effect of Hsp90 inhibition also reflected more strongly on the mature protein than on nascent EGFRvIII (Figure 5). This suggests that impact of CK2 inhibition on EGFRvIII protein levels may be at least partially explained by its effect on the chaperone system, and not due

to the many other effects of CK2 inhibition. These data complement my study using serine 13 mutants and strongly suggest that serine 13 phosphorylation has a positive role in EGFRvIII biogenesis, and that this phosphorylation serves to improve Cdc37's ability to recruit client to the Hsp90 complex. Furthermore, they answer the closely related third objective of my study, showing that CK2 activity does affect EGFRvIII stability and its interaction with the Cdc37/Hsp90 complex. Other than the defined objectives of this study, there are several additional novel results that warrant consideration. One of these is the apparent activity of nascent EGFRvIII.

Western blot analysis of immunoprecipitated nascent EGFRvIII revealed the presence of phosphorylation on Tyr1068 (Figure 4B). Following dimerization, EGFRvIII undergoes an autophosphorylation of specific sites (including Tyr1068) on its C-terminal tail, which, in turn, triggers downstream signaling cascades. Thus, the presence, of Tyr1068 phosphorylation, a major phosphorylation site on EGFR and EGFRvIII, is a marker for activity of the nascent receptor. Although phosphorylation was detected in immunoprecipitated nascent EGFRvIII, Western blotting of total cell lysates only showed phosphorylation at the molecular weight of mature EGFRvIII, not at the molecular weight of the Flag-detectable, nascent receptor (Figure 4A). This data suggests that EGFRvIII may dimerize to a small extent within the ER, however the proportion of active, nascent EGFRvIII is likely very small compared to that of mature receptor. Although the significance of the activity of this small population of nascent protein is not clear, to my understanding this is the first time that nascent EGFRvIII has been shown to activate within the ER. Although novel, this finding is not unprecedented, as one previous study showed that EGFR in the Golgi could activate. In this study, Dong *et al* have reported that EGFR may

become activated during transfer through the Golgi when co-expressed with a modified membrane-anchored ligand (Dong et al, 2005). These EGFR molecules migrated at a lower molecular weight when co-expressed with the modified ligand, apparently due to interference with addition and modification of complex carbohydrates within the Golgi.

Another interesting finding was on the effect of Cdc37 mutants on the cell cycle and Cdk4 expression. Cdk4 is a Cdc37/Hsp90 client protein that is overexpressed in approximately 50% of all glioblastomas and has been found necessary for EGFRvIII-induced gliomagenesis in mice with a p53-null background (Ortega et al, 2002; Holland et al, 1998). Although the reasons for cooperation between EGFRvIII and Cdk4 are not clear, targeting Cdc37 may be a means to regulate both client proteins and reduce the aggressiveness of the disease. Expression of Cdc37 WT and Cdc37 S13E caused a small 10% decrease in Cdk4 levels (Figure 6), and a slight (~5%) reduction in cells in the G1 phase of growth in low serum conditions (Figure 10B). The decrease in Cdk4 protein levels was unexpected because overexpression of Cdc37 in human prostate epithelial cells causes an increase in Cdk4 levels (Shwarze et al, 2003). One explanation for this is that another cochaperone besides Cdc37 is limiting for Cdk4 expression in U87MG-derived cells or that sufficient Cdc37 is already there to allow proper folding of all expressed Cdk4. If this were the case, overexpression of that cochaperone may increase Cdk4 protein levels. One candidate cochaperone is p23, which has been shown to interact with Cdk4, and bind to the Hsp90 complex in a mutually exclusive fashion from Cdc37 (Loubat, 1999; Pearl, 2005; Harst et al, 2005). In this case, overexpression of Cdc37 may compete with p23 for binding to the Hsp90/Cdk4 complex, reducing the stability of the late-stage Hsp90/ Cdk4 complex.

The slight, but statistically significant 5% decrease in G1-phase in cells transduced with Cdc37 WT and Cdc37 S13E indicated a slight growth advantage for the cells under low serum conditions (Figure 10B). The smallness of this change was unexpected considering the important roles that Cdc37 and Hsp90 play in promoting cell growth. Previous studies have shown that inhibiting Hsp90 induces G1 arrest in breast and colon cancer cell lines (Srethapakdi et al, 2000), and, similarly, reduction of Cdc37 levels by RNA interference in mammalian cells arrests cell growth in liver carcinoma cells (Pascale et al, 2005).

One explanation for the small change in cell growth comes from earlier reports on the role of EGFRvIII expression in proliferation of U87MG cells. Nishikawa *et al* have shown that overexpression of EGFRvIII in U87MG glioblastoma cells barely affected cell proliferation *in vitro*, but greatly enhanced their tumourigenicity *in vivo* (Nishikawa et al, 1994). For their *in vitro* study, they used both normal and reduced serum conditions to account for exogenous growth factors in the media that may mask the ligand-independent effects of EGFRvIII. They did not see any growth advantage conferred by EGFRvIII expression under normal serum, and only a slight advantage in low serum. As explanation, they suggested that since the U87MG cell line is a fully transformed cell line, further signaling by EGFRvIII may have little to no effect on the already signal-saturated system, and they conclude that the growth effects are dependent upon cell type and other mutational events. In agreement, a more recent study by Tang *et al* found that EGFRvIII expression in a non-tumourigenic murine hematopoietic cell line caused tumourigenicity *in vivo*, and increased proliferation *in vitro* (Tang *et al*, 2000). Nishikawa *et al* further argued that EGFR amplification and mutation occurs at a late stage of tumour development *in vivo*, possibly in response to selective pressures, such as ischemia, necrosis, and the requirement for

angiogenesis within a developed tumour. Such pressures do not exist *in vitro* and the benefits of EGFR amplification or mutation may not provide the same advantage. Indeed, an early study looking at EGFR amplification in cell lines derived from grade III and IV astrocytomas found EGFR amplification in only 1 of 22 cell lines despite such events being present in up to 50% of primary glioblastomas (Filmus et al, 1985). They concluded that EGFR amplification did not represent an advantage *in vitro*, and may even be detrimental in culture. These studies suggest that despite the small growth effects resulting from increased expression of the EGFRvIII caused by Cdc37 in my study, further study is warranted using *in vivo* models and *in vitro* studies using less aggressive cell lines.

A consideration for every Cdc37 client is that in rare cases, its chaperoning may be mediated by Cdc37 independently of Hsp90 (Rao et at, 2001; Kimura et al, 1997). In this case, Cdc37 can compensate for the absence of Hsp90 and stabilize the client protein on its own. I showed that neither the Cdc37 S13A or S13E mutant proteins could compensate for Hsp90 inhibition (Figure 7). In both cases, mature and nascent EGFRvIII were greatly destabilized by Hsp90 inhibition, thus demonstrating that Hsp90 is absolutely essential to EGFRvIII biogenesis and that Cdc37, alone, cannot offset for the loss of Hsp90 activity, regardless of phosphorylation status.

From a therapeutic perspective, the data in this study suggests that targeting Cdc37 or CK2 could be a means by which to control EGFRvIII signaling in glioblastoma. Currently, no drugs are available that specifically target Cdc37 function. Since Hsp90 is uncomplexed and inactive in normal cells (Kamal et al, 2003), the role of cochaperone Cdc37 may be minor as well, meaning a Cdc37 inhibitor may, possibly, have tumour-specific activity. Antisense approaches to targeting CK2 have shown high tumour specificity and would serve

as a means to prevent Cdc37 phosphorylation. Targeting CK2 would simultaneously disrupt the activation of molecules downstream of CK2, as well as the many oncogenic clients of Cdc37 (Ahmad et al, 2005). Such strategies may have usefulness in treating many types of cancer due to their broad array of targets, and may be useful for assisting other antineoplastic agents.

While the primary objectives of this study focused on analyzing the role of Cdc37 and is phosphorylation in the biogenesis of EGFRvIII, the results of this study may be applicable to other clients as well. For example, Cdc37 phosphorylation may also play an important role in biogenesis ErbB2, which also interacts with the Cdc37/Hsp90 complex, and appears to require this activity following biogenesis on the ER (Xu et al, 2005). A positively charged region that is common between the kinase domains of both ErbB2 and EGFR was shown to be responsible for determining their binding to Cdc37/Hsp90. Comparison of the structure of this lobe in EGFRvIII with that of ErbB2 and EGFR, whose Cdc37-dependence has not been characterized, may prove interesting. In addition, consideration of other cochaperones in the biogenesis of EGFRvIII, and the dynamics of cochaperone-cochaperone relationships in the chaperoning process may provide useful information for design of effective therapeutics.

Cdc37 is not the first Hsp90 cochaperone known to play a role determining the fate of nascent proteins. Previous work has identified several notable cochaperones with important roles in ERAD that would be of interest to study as follow-up work relating to ER-mediated degradation of nascent EGFRvIII. One of these is P97/VCP, an AAA ATPase, which mediates the export of certain misfolded proteins from the ER (Ye et al, 2001). P97 was first identified as a cochaperone as a result of its presence in complex with

immunopurified Cdc37 (Prince et al, 2005-2). It has since been found to have an important role in the release of certain polyubiquitinated client proteins into the cytosol during ERAD (Ye et al, 2005). One of these proteins is the CFTRΔF508 (cystic fibrosis transmembrane regulator) mutant protein (Vij et al, 2006). CFTRΔF508 is a client protein of Hsp90 whose improper folding in the ER and preferential degradation through ERAD underlies the principle cause of cystic fibrosis (Loo et al, 1998). Vij *et al* showed that inhibition of p97 expression by RNAi leads to selective inhibition of ERAD and accumulation of CFTRΔF508 in the ER (Vij et al, 2006). Due to its association with Cdc37, and a proven role in ERAD, p97 could potentially have an important role in mediating degradation of EGFRvIII.

Another potential protein of interest in regulating degradation of nascent EGFRvIII is the E3 ubiquitin ligase/ Hsp90 cochaperone CHIP (carboxyl terminus Hsc70-interacting protein). CHIP has been shown to mediate the degradation of transmembrane Hsp90 clients that mature through the ER, including CFTRΔF508 (Younger et al, 2004) and ErbB2 (although its interaction with the nascent ErbB2 protein has not been specifically examined) (Zhou et al, 2003; Xu et al, 2003). The binding of CHIP to the Hsp90 complex is particularly interesting because it negatively regulates Hsp90's function, promoting degradation of the Hsp90 client, and is known to remodel the Hsp90 complex by excluding the cochaperone p23 to prevent protein folding (Connell et al, 2001). Due to its role in promoting degradation of transmembrane Hsp90-client proteins, including fellow tyrosine kinase ErbB2, and because of its ability to compete with other cochaperones for binding, a role for CHIP in EGFRvIII degradation is also plausible and warrants investigation. Specifically, the effect, if any, that Cdc37 has on regulating the binding of CHIP to EGFRvIII in the ER would be a relevant consideration with respect to this thesis.

Recently, Aha1, the only known cochaperone to activate the Hsp90 ATPase, was surprisingly shown to have a negative effect on the stability of CFTRΔF508 in the ER (Wang et al, 2006). Knockdown of Aha1 expression by RNAi rescued misfolded CFTRΔF508 from degradation, while knockdown of cochaperones p23 and FKBP8 more predictably decreased its stability. Wang *et al* suggested that removing Aha1 from the cell might alter the cochaperone composition of the Hsp90/ CFTRΔF508 complex to something that is more supportive towards the folding of the mutant protein. Meanwhile, p23 and FKBP8 may be more essential components to such a complex, and their loss results in less favourable folding energetics and more client degradation. To conclude, they suggest that the energetics of folding likely varies between client proteins and cell lines, and, therefore, the cellular environment determines the difference between a deleterious mutation and a tolerated one. Thus, altering this cochaperone balance may be a means by which to impose a level of control over other conformationally altered or mutated proteins, like EGFRvIII. Close examination of the roles of these cochaperones may therefore provide important targets for the treatment of abnormal proteins at the root of diseases such as glioblastoma and cystic fibrosis.

In summary, Cdc37 and its phosphorylation appear to play important roles in promoting biogenesis of EGFRvIII. It appears that Cdc37 is a limiting factor in EGFRvIII maturation, and its absence or inactivity may limit recruitment to Hsp90 and result in degradation through ERAD. Inhibition of CK2, the only kinase known to phosphorylate Cdc37 serine 13, also reduced the affinity of Cdc37 and Hsp90 for nascent EGFRvIII, and reduced levels of mature EGFRvIII protein. Taken together, these data strongly suggest an important role for Cdc37 and CK2 in biogenesis of EGFRvIII within tumours, and identify

both of these proteins as intriguing therapeutic targets for disrupting EGFRvIII in the treatment of glioblastoma.

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