*Oncogene*. Author manuscript; available in PMC 2015 July 02.

Published in final edited form as:
*Oncogene*. 2015 January 2; 34(1): 15–26. doi:10.1038/onc.2013.519.

# Restricting direct interaction of CDC37 with HSP90 does not compromise chaperoning of client proteins

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## Abstract

The HSP90 molecular chaperone plays a key role in the maturation, stability and activation of its clients, including many oncogenic proteins. Kinases are a substantial and important subset of clients requiring the key cochaperone CDC37. We sought an improved understanding of protein kinase chaperoning by CDC37 in cancer cells. CDC37 overexpression in human colon cancer cells increased CDK4 protein levels, which was negated upon CDC37 knockdown. Overexpressing CDC37 increased CDK4 protein half-life and enhanced binding of HSP90 to CDK4, consistent with CDC37 promoting kinase loading onto chaperone complexes. Against expectation, expression of C-terminus truncated CDC37 (ΔC-CDC37) that lacks HSP90 binding capacity did not affect kinase client expression or activity; moreover, as with wildtype CDC37 overexpression, it augmented CDK4-HSP90 complex formation. However, although truncation blocked binding to HSP90 in cells, ΔC-CDC37 also showed diminished client protein binding and was relatively unstable. CDC37 mutants with single and double point mutations at residues M164 and L205 showed greatly reduced binding to HSP90, but retained association with client kinases. Surprisingly, these mutants phenocopied wildtype CDC37 overexpression by increasing CDK4-HSP90 association and CDK4 protein levels in cells. Furthermore, expression of the mutants was sufficient to protect kinase clients CDK4, CDK6, CRAF and ERBB2 from depletion induced by silencing endogenous CDC37, indicating that CDC37's client stabilising function cannot be inactivated by substantially reducing its direct interaction with HSP90. However, CDC37 could not compensate for loss of HSP90 function, showing that CDC37 and HSP90 have their own distinct and non-redundant roles in maintaining kinase clients. Our data substantiate the important function of CDC37 in chaperoning protein kinases. Furthermore, we demonstrate that CDC37 can stabilise kinase clients by a mechanism that is not dependent on a substantial direct interaction



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**Conflict of interest:** PW has received research funding on HSP90 inhibitors from Vernalis and intellectual property from this collaboration has been licensed to Vernalis and Novartis. PW has been a consultant to Novartis.


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between CDC37 and HSP90, but nevertheless requires HSP90 activity. These results have significant implications for therapeutic targeting of CDC37.

**Keywords**

CDC37; HSP90; client; kinase; chaperone



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## Introduction

Client protein chaperoning by HSP90 requires a specialised cohort of cochaperones (1). Through the formation of transient complexes, these cochaperones participate in progression of the ATP-dependent chaperone cycle and maturation of client proteins to enable their eventual functional activities (2). CDC37 acts predominantly as a kinase-specific cochaperone. The yeast CDC37 homologue is essential for viability (3) and is estimated to regulate three quarters of the kinome (4). Similarly in mammalian cells, CDC37 interacts with a large proportion of kinases (5) and in concert with HSP90 directs the maturation of numerous oncogenic clients (6-12). Although the precise mechanisms by which CDC37 facilitates the actions of HSP90 are not fully understood, it has been proposed that association of CDC37 with the N-terminus of HSP90 blocks its ATPase activity and helps kinase client loading before progression of the chaperone cycle (2;13;14).

CDC37 may also have the capacity to promote maturation of kinase clients independent from HSP90 (15;16). In *Saccharomyces cerevisiae*, the kinase-binding domain of Cdc37 alone stabilised v-Src to some degree, indicating that it may function without direct HSP90 interaction, albeit less efficiently (17). Similar truncated Cdc37 mutants were also sufficient to maintain the viability of *Schizosaccharomyces pombe* (18). The mechanism of this seemingly HSP90-independent activity is unknown and it is uncertain whether it is conserved in human CDC37. Against this notion is that C-terminally truncated mammalian CDC37 mutants deficient in binding to HSP90 were reported to reduce client activity (7;19) and decreased the expression of CRAF and CDK4 in normal prostate cells (20).

CDC37's ability to regulate the chaperoning of several clients that promote cell cycle progression and survival suggests that it may be particularly important in cancer cells (21;22). In support of this, transgenic mice overexpressing CDC37 show an increased incidence of tumours (23;24). Furthermore, CDC37 may contribute towards the initiation of prostate cancer (24). We have previously shown that CDC37 knockdown causes depletion of kinase client proteins as well as growth inhibition in several cancer cell lines (25). Similarly, CDC37 silencing in prostate cancer cells reduced signaling through clients and induced growth arrest (26). Therefore, targeting CDC37 could represent a strategy for disrupting multiple kinase-dependent oncogenic signaling pathways that sustain malignancy (27;28).

Here we set out to understand further the functional role of CDC37 in human cancer cells. Complementing our previous knockdown approach (25), CDC37 overexpression was used to explore the extent to which selected protein kinase clients were dependent on CDC37 levels for their chaperoning. Furthermore, by investigating the effects of CDC37 mutants that disrupt the interaction with HSP90, we demonstrate that CDK4 protein stability is

particularly dependent on the level of CDC37 but that substantial direct interaction of CDC37 with HSP90 is not required for efficient chaperoning of kinase clients. These results help to distinguish between the functions of CDC37 and HSP90, and have significant mechanistic and therapeutic implications.

## Results

### CDC37 overexpression increases cellular levels of client CDK4

To complement our previous CDC37 knockdown studies in HCT116 and HT29 colon cancer cells (25), we generated isogenic cell lines stably overexpressing CDC37 (Figure 1a). Two clones were chosen from each cell line, which expressed at least 8-fold (by densitometry of western blots; Supplementary Figure S1) more CDC37 compared to parental and empty vector-transfected cells (Figure 1a). The exogenous CDC37 was phosphorylated at Ser13 (Figure 1a, quantified in Supplementary Figure S2) and therefore activated (29). Although our siRNA silencing studies showed that CDC37 is required to maintain expression of these kinase clients (25), CDC37 overexpression did not affect cellular CRAF, AKT or phosphorylated AKT protein levels (Figure 1b, quantified in Figure 1c). However, significant elevation of CDK4 protein was observed in all HCT116 and HT29 clones that overexpressed CDC37 (Figure 1b, quantified in Figure 1c). In CDC37-overexpressing clone C1, CDK4 levels were reduced to basal levels by silencing CDC37 (siRNAs O3 and O4, Figure 1d). These data strongly suggested that constitutive CDC37 levels are rate-limiting for the expression of CDK4, but not the other kinases studied.

Since HSP90 chaperoning aids the formation of active signaling-competent proteins (30), we next addressed whether increasing CDC37 levels promoted the signaling output from its clients. RAF kinase activity, measured by phosphorylated MEK1/2 and ERK1/2 levels, was unaltered in CDC37-overexpressing clones compared to empty vector controls (Figure 2a, quantified in Supplementary Figure S3). Despite elevated CDK4 expression, RB phosphorylation at S795 and S780 remained unchanged when CDC37 was overexpressed and Cyclin D1 expression was unaltered (Figure 2a). Since other factors may limit CDK4 activity in intact cells, we performed a kinase assay with CDK4 immunoprecipitates to determine whether CDC37 overexpression increased intrinsic CDK4 activity. HCT116 and HT29 CDC37-overexpressing clones did not show different levels of RB phosphorylation activity compared to controls (Figure 2b). Therefore, although basal CDC37 levels were rate-limiting for CDK4 protein expression, they did not restrict the kinase activity of its clients.

### CDC37 overexpression promotes CDK4 stabilisation

As elevated CDK4 levels in CDC37-overexpressing cells may be due to its increased stability, we assessed CDK4 protein half-life. CDC37-overexpressing clone C8 (HT29) showed a slower rate of CDK4 degradation following treatment with the protein synthesis inhibitor cycloheximide compared to the empty vector control (Figure 3a). Since CDK4 stability is influenced by binding partners such as D Cyclins that have relatively short half-lives (31), we also performed a pulse chase experiment to avoid potential artefacts resulting

from blocking protein synthesis. CDK4 half-lives were 3.3h in the empty vector clone, and 9.7h in the CDC37-overexpressing clone (Figure 3b), an almost 3-fold increase.

To determine whether the dependence of CDK4 on CDC37 might be conserved with other close CDK family members, CDK6 was examined. CDK6 levels were unchanged in CDC37-overexpressing clones (Figure 3c). Consistent with this finding, CDK6 protein half-life was similar in the empty vector control and CDC37-overexpressing clone (4.4h compared with 5.6h, respectively; Figure 3d). Therefore, the dependence of CDK4 on CDC37 was not shared by CDK6.

### CDC37 overexpression promotes CDK4 binding to HSP90

We hypothesised that the increased CDK4 stability in CDC37-overexpressing cells was due to enhanced binding to HSP90. By immunoprecipitation we showed that more CDK4 was associated with HSP90 in CDC37-overexpressing HCT116 and HT29 cells than in the empty vector clones (Figure 4a, quantified in Supplementary Figure S4a). Less HSP90 was immunoprecipitated with CDK4 in HT29 cells than HCT116 cells, which is most likely due to lower expression of HSP90 and CDC37 in this cell line as shown in Supplementary Figure S4b. Although undetectable in HT29 cells, the level of Cyclin D1 associated with CDK4 was unaltered in HCT116 cells (Figure 4a).

Further analysis of protein complexes by gel filtration indicated that in control cells, CDK4 peaked in three main forms: ~44kd, ~440kd and large complexes of >640kd (Figure 4b, top). Cyclin D1 segregated into complexes of <440kd, whereas HSP90 and CDC37 were most abundant in complexes of ~640kd and ~440kd, respectively. Interestingly, CDC37-overexpressing cells exhibited a different distribution of CDK4 whereby the kinase was mostly present in higher molecular weight complexes containing HSP90 and CDC37, whereas the fractions in which HSP90, CDC37 and Cyclin D1 were detected were comparable with the vector control (Figure 4b, bottom). These data are consistent with CDC37 promoting CDK4 recruitment to large HSP90 chaperone complexes.

### ΔC-CDC37 blocks HSP90 binding but enhances CDK4-HSP90 association

Since our findings so far were indicative that CDC37 supports client protein stabilisation by promoting binding to chaperone complexes, we next hypothesized that this required interaction between CDC37 and HSP90. To test this hypothesis we expressed CDC37 protein truncated after aa173 (ΔC-CDC37), thereby lacking the HSP90-interacting C-terminal domain (14) and blocking binding to HSP90 (17). ΔC-CDC37 was detected in transfected HCT116 and HT29 clones, although at a lower level than the endogenous CDC37, and was phosphorylated at Ser13 (Figure 5a). Against expectation, expression levels of client proteins CDK4, CRAF, BRAF and AKT were unchanged in cells that expressed ΔC-CDC37 (Figure 5a, quantified in Supplementary Figure S5). Downstream signaling output from CDK4 and RAF clients, as determined by phosphorylated ERK and RB respectively, revealed no changes, nor was there any alteration in AKT phosphorylation (Figure 5b). Since the presence of endogenous CDC37 could mask the ΔC-CDC37 phenotype, we utilised siRNAs that silenced either the endogenous CDC37 only (O4: Figure 5c) or both forms (O3: Figure 5c). Knockdown of endogenous CDC37 when ΔC-CDC37

was expressed resulted in clients CDK4 and CRAF being partially depleted to the same extent as observed by knockdown of both endogenous and ΔC-CDC37 (Figure 5c). Therefore, ΔC-CDC37 failed to act as a dominant negative with respect to client stabilisation.

Gel filtration separation of protein complexes in HCT116 and HT29 clones indicated that ΔC-CDC37 was present in much smaller molecular weight complexes than endogenous CDC37 (Figure 6a). HSP90 was not detected in the same fractions as ΔC-CDC37, consistent with ΔC-CDC37 being impaired in binding HSP90. In contrast, some CDK4 was found in the same fractions as ΔC-CDC37, suggesting that they could coexist in a complex (Figure 6a). Interestingly, both ΔC-CDC37-expressing clones exhibited a shift of endogenous CDC37 and CDK4 to larger complexes, possibly indicating an increase in HSP90-endogenous CDC37-client complexes (Figure 6a). Supporting this, increased binding of CDK4 to HSP90 was confirmed by immunoprecipitation of CDK4 from ΔC-CDC37-expressing clones (Figure 6b, c, quantified in Supplementary Figure S6). In HCT116 cells the increased binding of CDK4 to HSP90 was more apparent in clone T11 than T9, consistent with higher expression of ΔC-CDC37 in clone T11 than T9 (Figure 5a, b). Therefore, contrary to our original hypothesis that CDC37-HSP90 binding deficiency would compromise the chaperoning of clients, the formation of CDK4-HSP90 complexes was actually enhanced.

### ΔC-CDC37 shows decreased binding to clients and reduced stability

As CDC37 forms functional dimers (13;14;32), we next questioned whether ΔC-CDC37 heterodimerised with endogenous CDC37 in HCT116 cells, as this could afford an increased functionality to ΔC-CDC37. Following dual expression of HA- or FLAG-tagged CDC37 and ΔC-CDC37, we looked for wildtype and truncated CDC37 in FLAG immunoprecipitates (Supplementary Figure S7a). No HA-ΔC-CDC37 was detected in a FLAG-CDC37 pulldown, nor was HA-CDC37 observed in a FLAG-ΔC-CDC37 pulldown, suggesting that ΔC-CDC37 does not dimerise with wildtype CDC37. Thus heterodimerisation is unlikely to be a complicating factor in assessing the ΔC-CDC37 phenotype.

Next, to determine whether the C-terminal deletion affected client binding, we expressed FLAG-tagged CDC37 or ΔC-CDC37 and analysed FLAG immunoprecipitates. Firstly, we confirmed that ΔC-CDC37 did not associate with HSP90; however, increased binding to HSC70 (HSP70) was seen (Supplementary Figure S7b). Interestingly, much less CDK4 or CRAF were bound to FLAG-ΔC-CDC37 compared to FLAG-CDC37 (Supplementary Figure S7b). This finding may indicate that in the absence of a direct interaction with HSP90, CDC37 has a lower affinity for client proteins or that the C-terminus of CDC37 is involved in binding clients, as suggested by others (33).

Since removal of the C-terminus may influence the overall structure and/or stability of CDC37, we determined the half-life of ΔC-CDC37 using a post-cycloheximide time course. As shown in Supplementary Figure S7c, ΔC-CDC37 degraded rapidly in HCT116 and HT29 cells and has a much shorter half-life than endogenous CDC37, consistent with lower expression levels of the mutant form (Figure 5a). Together with the decreased binding to

kinase clients, the instability of the C-terminal deletion mutant likely had detrimental effects in addition to the intended loss of HSP90 binding. Thus we subsequently sought additional, more specific mutants to model inhibition of CDC37-HSP90 association.

## CDC37 point mutations reduce binding to HSP90

Appropriate mutations were chosen based on the structure of CDC37 in complex with HSP90. The electron microscopy-derived model of a CDC37-CDK4-HSP90 complex (Figure 7a) and CDC37-HSP90 X-ray crystal structure (Figure 7b) indicate how the N-terminal domains of HSP90 dimers interact with the CDC37 C-terminal domain. Several critical residues are involved in a hydrophobic interaction: we chose to modify residues M164 and L205, which from a model of the CDC37-HSP90 interface (Figure 7c) were predicted to disrupt the protein-protein interaction without compromising the rest of the protein. Both residues were substituted with either alanine as a small residue, or arginine to introduce a positive charge, and the corresponding single and double mutants generated.

FLAG-tagged wildtype or mutant CDC37 constructs were transfected into HCT116 and HT29 colon cancer cells, giving higher expression than the endogenous CDC37 (HCT116 shown in Figure 7d and 12-fold overexpression estimated for mutant M164A in Supplementary Figure S8). To determine how the mutations affected chaperone complexes, FLAG immunoprecipitates were analysed. As predicted, in both HCT116 and HT29 cells, less HSP90 associated with all the mutants compared to wildtype CDC37 (Figure 7e). Of note, quantitation indicated that M164R/L205R was partially effective at blocking HSP90 association whilst mutants M164A and L205A disrupted the interaction by 94% and 90%, respectively (Figure 7f). As further confirmation, ITC measurements with purified recombinant proteins gave $K_d$ values of 2.26μM and 7.19μM for the binding to HSP90β of FLAG-tagged wildtype and M164A, respectively, and the FLAG-CDC37 M164R/L205R double mutant showed no detectable binding. The differences in the binding observed for purified recombinant protein and intact cell settings could be due to effects of additional proteins in cellular chaperone complexes. However, the key point is that the CDC37 mutants were shown to exhibit reduced HSP90 binding in cells, which was substantial and almost complete in some cases.

As expected and in contrast to ΔC-CDC37 (Supplementary S7b), client proteins CDK4 and CDK6 associated with all of the CDC37 point mutants to the same extent as wildtype CDC37 in cells (Figure 7e). Interestingly, CDC37 M164A/L205A and all the arginine-substituted mutants showed greater binding to CRAF, particularly in HT29 cells (Figure 7e) but consistent with basal CDC37 activity not being rate limiting for CRAF in these cells lines (Figure 1b, c) this did not affect CRAF expression levels.

## CDC37 point mutants retain the ability to promote chaperoning of CDK4

To determine whether the HSP90 binding-impaired CDC37 mutants compromised client protein stability, we analysed the expression level of several clients. Firstly, we confirmed that all CDC37 mutants were phosphorylated (Figure 8a). Next, we found no change in ERBB2, CRAF or AKT expression following overexpression of the CDC37 mutants, as also

observed with wildtype overexpression (Figure 8a, CRAF quantified in Supplementary Figure S9).

For subsequent studies we primarily focussed on CDC37 point mutants M164A, L205A and M164R/L205R that most effectively blocked binding to HSP90 (in cells by immunoprecipitation or with recombinant proteins by ITC). These CDC37 mutants did not affect the signalling output of kinase clients, as determined by phosphorylation of AKT and ERK1/2 in HCT116 (Figure 8b).

In agreement with our earlier findings (Figure 1a), CDK4 expression was elevated in cells overexpressing FLAG-tagged wildtype CDC37 (Figure 8a, quantified in Supplementary Figure S9). Surprisingly, instead of compromising the chaperoning of this client as predicted, CDK4 levels were elevated similarly to wildtype when any of the mutants were expressed (Figure 8a, quantified in Supplementary Figure S9). Furthermore, CDK4 immunoprecipitates from all mutant CDC37-expressing cells showed increased CDK4-HSP90 association similar to that seen with wildtype FLAG-CDC37 overexpression (Figure 8c, quantified in Supplementary Figure S10). Thus expressing HSP90 binding-impaired CDC37 point mutants unexpectedly phenocopied wildtype CDC37 overexpression.

A possible explanation for the ability of the CDC37 point mutants to promote the chaperoning of CDK4, despite substantially compromised interaction with HSP90, could be the formation of mutant-wildtype CDC37 dimers that may account for the small amount of HSP90 detected in immunoprecipitates of the selected CDC37 mutants (Figure 7e). However, specific silencing of endogenous CDC37 expression using 3′ UTR-targeted siRNA before immunoprecipitation of CDC37 M164A or L205A failed to reduce HSP90 binding further when compared with FLAG-CDC37 (Figure 8d). Furthermore, CDK4 association with the CDC37 mutants was also unaffected by endogenous CDC37 knockdown. Thus the unexpected retention of chaperoning activity by the CDC37 point mutants cannot be attributed to heterodimerisation with endogenous CDC37.

## CDC37 point mutants can replace the chaperoning role of endogenous CDC37 but not HSP90

Our findings so far indicated that the CDC37 point mutants that block HSP90 interaction act similarly to wildtype CDC37 in promoting the chaperoning of CDK4. We rationalised that the mutants could potentially replace the chaperone function of endogenous CDC37. To test this, we silenced endogenous CDC37 and examined the effect on kinase client proteins. As shown in Figure 9a, CDC37 knockdown in parental HCT116 cells caused the expected depletion of CDK4, CDK6, CRAF and ERBB2 (25). High-level expression of FLAG-CDC37 rescued depletion of kinase proteins following exposure to siRNA that silenced wildtype CDC37 (Figure 9a, quantified in Supplementary Figure S11). Importantly, expression of CDC37 M164A or L205A mutants was also sufficient to protect HSP90/CDC37 clients CDK4, CDK6, CRAF and ERBB2 from depletion by CDC37 knockdown (Figure 9a, quantified in Supplementary Figure S11). These results suggested that despite a severely restricted capacity to bind HSP90, the CDC37 point mutants were nevertheless still functional in maintaining client protein stability in cells.

Assaying kinase activity following knockdown of endogenous CDC37 demonstrated that CDK4 function was not affected by expression of the point mutants, as also seen with wildtype CDC37 overexpression (Figure 9b). This is consistent with CDC37 primarily affecting the stability rather than the activity of CDK4.

The hypothesis that CDC37 may chaperone clients independently of HSP90 led us next to determine if the CDC37 mutants could compensate for loss of HSP90 activity. We found that partial knockdown of HSP90α and HSP90β resulted in a reduction of client proteins such as CDK4 and ERBB2 (Figure 9c). However, overexpression of wildtype CDC37 or mutants M164A or L205A did not lessen the extent of client protein depletion upon HSP90 silencing. In addition, we found that CDC37 overexpression rescues the sensitisation to pharmacological HSP90 inhibitors that we previously observed (25) upon silencing of CDC37, and that overexpression of CDC37 mutants M164A, L205A or M164R/L205R has the same protective effect (Table 1). Moreover, overexpression of wildtype or mutant CDC37 did not result in cellular resistance to the HSP90 inhibitor 17-AAG compared to the parental cells. Together, these results demonstrate that CDC37 cannot substitute for HSP90 in chaperoning kinase clients and both must play an important role, even though significant direct binding between the two is not required.

## Discussion

To complement our previous siRNA silencing studies showing that several HSP90 clients were dependent on CDC37 in cancer cells (25), here we used an overexpression and mutation approach to further investigate the role of CDC37 in client stabilisation, as well as specifically exploring the importance of CDC37's direct interaction with HSP90. We found that overexpressing wildtype CDC37 in human colon cancer cell lines HCT116 and HT29 had no effect on the expression and signalling output of all but one of the representative CDC37 clients studied, the only exception being a clear increase in CDK4 protein levels. Our results are in contrast to those in normal prostate epithelia in which CDC37 overexpression promoted the activity of clients (20). These differences may be rationalised by the elevation in CDC37 levels seen in several cancers (24;34;35), which could account for high CDC37 activity in the cancer cell lines we studied, so that increased expression had no further effect. Since the chaperoning of kinase clients is mediated by a multi-protein complex including HSP70, HSP90, HSP40, CDC37 and CK2 (36), other components could also become limiting when CDC37 is overexpressed.

We demonstrate that CDC37 has an important role in maintaining CDK4 stability in colon cancer cells and furthermore that binding of CDK4 to HSP90 is increased by CDC37 overexpression, consistent with a model in which CDC37 promotes client association with the chaperone (6;13). It is uncertain why the basal expression levels of CDC37 were rate-limiting for this client but not others; differences in the degree of dependence on CDC37 by particular client proteins were also shown in our CDC37 siRNA knockdown studies (25). The strong dependence of CDK4 on CDC37 is consistent with the close correlation between CDC37 and Cyclin D1 expression in highly proliferative tissues (6). We did not observe a concomitant increase in stability of a closely related CDK, CDK6, in CDC37-overexpressing cells; this could be due in part to the stronger association of CDC37 with

CDK4 compared to CDK6 (6). Despite elevated CDK4 levels, neither its kinase activity nor active CDK4/Cyclin D complexes were increased in CDC37-overexpressing cells. CDC37 and Cyclin D1 overexpression collaborate in tumourigenesis (23;34), which could suggest that in our models Cyclin D1 levels may become limiting for CDK4 kinase activity when overexpressing CDC37.

In addition to its cochaperone role, yeast Cdc37 was suggested to have its own chaperoning capacity separate from HSP90 (15). Evidence for such independent activity with human CDC37 was previously lacking. In yeast, Cdc37 exists predominantly free from HSP90 complexes (18;37); thus the likelihood of Hsp90-independent functionality may be greater than for human CDC37 which binds HSP90 more tightly (13). Indeed, expression of an HSP90 binding-impaired C-terminally truncated CDC37 in non-immortalised human prostate cells blocked CDC37 activity leading to loss of kinase client proteins, growth arrest and apoptosis (20). The same CDC37 mutant also exhibited dominant negative effects on the client CRAF in insect cells (7), suggesting that direct interaction with HSP90 was essential for CDC37 function. To further investigate CDC37's potential HSP90-independent activity in human tumour cells we expressed a similar C-terminal truncation mutant (17) in the HCT116 and HT29 colon cancer lines in which we had previously characterised the phenotypes of CDC37 knockdown (25) and overexpression (herein). Surprisingly, although we confirmed that truncation prevented HSP90 binding, ΔC-CDC37 expression did not alter the levels or activity of clients defined by RNAi to be CDC37-dependent (25) and therefore did not act as a dominant negative. On the contrary, an unexpected increase in CDK4-HSP90 complexes and shift towards higher molecular weight HSP90 chaperone complexes suggested that ΔC-CDC37 could promote the recruitment of clients to HSP90. However, clients CDK4 and CRAF associated less with ΔC-CDC37 compared to wildtype CDC37, an observation also made for the interaction of a similar truncated CDC37 mutant with a mutant HCK client (38). Furthermore, in contrast to wildtype CDC37 overexpression, ΔC-CDC37 did not cause an elevation in CDK4 levels, perhaps due to low expression of the mutant. We concluded that using a C-terminal deletion approach to block CDC37's interaction with HSP90 is limited owing to the reduced interaction with kinase clients and instability of the mutant protein.

We reasoned that examining the importance of CDC37-HSP90 association required more subtle mutation analysis and so generated a series of CDC37 point mutants substituted at residues M164/L205 that are involved in HSP90 binding (14;39). Mutation of these two sites was previously found to be efficient at blocking the interaction between CDC37 and HSP90 in living cells (40). These studies and our own binding data provided validation for the use of the selected point mutants to inhibit CDC37-HSP90 association in our cancer cell models.

Surprisingly, instead of having a dominant negative effect on the chaperoning of CDC37 clients, we found that M164 and L205 mutants, that showed substantially reduced binding to HSP90, were able to phenocopy wildtype CDC37 overexpression. In agreement with our findings, another CDC37 mutant deficient in HSP90 binding failed to perturb the activity of a mutant HCK client in human cells, and in fact modestly increased its kinase activity and augmented binding of the client to HSP90 (38). In our own studies, an increase in CDK4

expression and CDK4-HSP90 association, comparable to wildtype, was apparent when the CDC37 point mutants were expressed. Furthermore, the HSP90 binding-impaired mutants were able to substitute for endogenous CDC37 in maintaining the stabilisation of client proteins CDK4, CDK6, CRAF and ERBB2, again similar to overexpressed wildtype CDC37. These findings were indicative of substantial direct CDC37-HSP90 binding mediated by the C-terminus of CDC37 with N-terminus of HSP90 not being essential for CDC37 to chaperone kinase clients in cells.

Although CDC37 may possess activity when free from HSP90 complexes (15), we found evidence that CDC37 and HSP90 do not have completely redundant roles, since CDC37 overexpression could not compensate for the effects of HSP90 knockdown or treatment with an HSP90 inhibitor. One possibility is that the two proteins have separate but sequential roles in kinase client maturation.

A current model for CDC37's cochaperone role involves its interaction with the N-terminus of HSP90 transiently inhibiting the HSP90 ATPase cycle, thereby permitting the loading of client proteins (14, Figure 10; Model A). Our results indicate that, at least under conditions where direct CDC37-HSP90 binding is severely restricted, CDC37 can efficiently maintain the maturation of clients by promoting the association of kinase clients with HSP90 through an alternative mechanism (Figure 10; Model B). Since kinase clients may interact with HSP90 in a specific orientation, as suggested for CDK4 in which the two lobes of the kinase bind to separate domains of HSP90 allowing conformational changes to be relayed during the chaperone cycle (41), one speculative role for CDC37 could be to present clients in a conformation that is more amenable to HSP90 association. It is unclear whether CDC37 possesses intrinsic chaperoning activity or acts more as a scaffold that protects clients from degradation prior to chaperoning by HSP90, which due to their inherent instability may be particularly important for immature protein kinases (42). Elucidation of the precise mechanism by which CDC37 and HSP90 are able to chaperone kinase clients is of considerable interest. This will best be addressed by extensive proteomic and structural biology studies.

The largely kinase-specific clientele of CDC37 presents an opportunity to target selectively an important oncogenic subset of HSP90 clients (21;28). So far, specific inhibitors of CDC37 have not been reported. Natural product inhibitors celastrol and withaferin A disrupt the interaction between HSP90 and CDC37 and show anticancer activity (43;44), although this has not been linked directly to CDC37 blockade, particularly in the case of celastrol, which elicits its growth inhibitory effect via multiple mechanisms (45). While our data support CDC37 as a potential cancer drug target, importantly, our results with M164/L205 mutants indicate that substantially disrupting the CDC37-HSP90 protein interaction mediated by this C-terminal region of CDC37 is not sufficient to prevent the cochaperone from promoting HSP90-client association. This suggests that therapeutic alternatives to blocking CDC37-HSP90 association are needed. Possible strategies could involve targeting other CDC37 interactions, for example homodimerisation or client binding (46). CDC37's N-terminal region is implicated in binding to kinases (47); however, other regions may also influence the interaction (33). Phosphorylation/dephosphorylation of CDC37 is another important regulatory process that could be targeted (48;49). Further research into these other

aspects of CDC37 regulation will be required to define druggable means for inhibiting CDC37, as well as to more completely understand the chaperone role of CDC37 in cancer and normal cells.

## Materials and methods

### Cell culture

HCT116 and HT29 human colon cancer cell lines were obtained from ATCC and cultured in DMEM (Sigma-Aldrich, Gillingham, UK) supplemented with 10% FCS (PAA Laboratories, UK), 2mM L-glutamine and non-essential amino acids (Invitrogen, Paisley, UK). Cells were maintained at 37°C, 5% $CO_2$ in a humidified incubator.

### Plasmid cloning and transfection

See supplementary information for primer and oligo sequences. CDC37 open reading frame was amplified by RT-PCR from human MCF-7 cDNA using primers P1/P2. The C-terminal truncation mutant was generated with primers P1/P3 which introduced a stop codon after aa173. Products were blunt cloned into pPCR-Script Amp (Stratagene, Agilent Technologies, Stockport, UK) or pCR4Blunt TOPO (Invitrogen) and fully sequenced. For point mutations the QuikChange Multi Site-directed mutagenesis kit (Stratagene) was used with primers P4-P7. CDC37 constructs were transferred to bicistronic eukaryotic expression vectors pEFIRES-P or pEFIRES-N (50) , N-terminal tags being added subsequently. Transfection of supercoiled plasmid DNA was performed with Lipofectamine 2000 (Invitrogen). Stable transfectants were selected with 3μg/ml puromycin (Sigma-Aldrich) and 1mg/ml G418 (Invitrogen; dual construct transfections only).

### siRNA transfection

Transfection of 20nM CDC37 or control siRNAs was performed using Oligofectamine (Invitrogen) according to the manufacturer's protocol. Hiperfect (Qiagen) reverse transfection protocol was used for 100nM HSP90α and HSP90β or corresponding inactive siRNAs. Sequences are detailed in supplementary information.

### Western blotting and immunoprecipitation

As described previously (25). Blots shown are representative of at least 2 independent experiments. Antibodies and densitometry are detailed in supplementary information.

### Gel filtration

Cell pellets were resuspended in filtered lysis buffer (50mM Tris pH 7.6, 150mM NaCl, 0.5% NP40, 1mM DTT) with complete protease inhibitor cocktail (Roche, Switzerland) and incubated on ice for 30 min. Samples were centrifuged at 17,000 × g for 30 min at 4°C, soluble lysates removed and centrifuged at 17,000xg until completely clear. 3mg protein was run through a 25ml Superose12 gel filtration column using an AKTA Chromatography system (GE Pharmacia, Amersham, UK), collecting 0.5ml fractions.




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### Pulse chase

As described previously (25).

### Isothermal titration calorimetry and $K_d$ determinations

Detailed in supplementary information.

### Growth inhibition assay

Sulphorhodamine B assay was performed as previously (25).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

We thank our colleagues in the Signal Transduction and Molecular Pharmacology Team and Chaperone Project Team for helpful discussions and Craig McAndrew for valuable assistance with gel filtration. The authors' work is supported by Cancer Research UK Programme grant number C309/A8274 and C309/A11566. PW is a Cancer Research UK Life Fellow.

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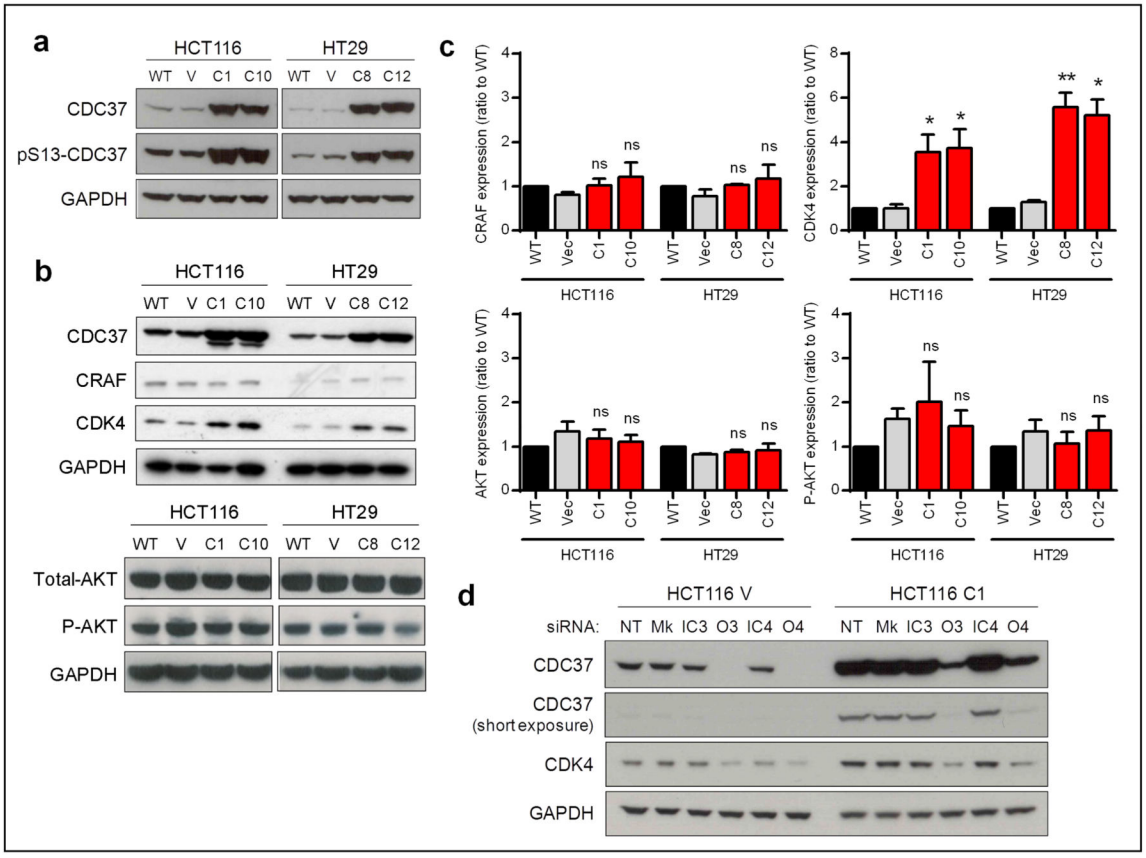


**Figure 1. Kinase client levels following CDC37 overexpression**

(a) Western blot showing total CDC37 and phospho-S13-CDC37 expression in wildtype (WT), empty vector-transfected (V) or CDC37-overexpressing HCT116 (clones C1 and C10) and HT29 (clones C8 and C12) colon cancer cells. The level of total CDC37 overexpression is quantified in Supplementary Figure S1 and phospho-S13-CDC37 is quantified in Supplementary Figure S2. (b) Expression of CDC37, CRAF, CDK4, total AKT and S473-phosphorylated AKT in wildtype (WT), empty vector-transfected (V) or CDC37-overexpressing HCT116 (C1, C10) and HT29 (C8, C12) cells. (c) Client protein expression in wildtype (WT), empty vector-transfected (Vec) or CDC37-overexpressing HCT116 (C1, C10) and HT29 (C8, C12) cells quantified by densitometry from western blots. Represented as mean±SE ratios relative to the wildtype cells from at least 3 independent experiments. Statistical analysis was performed using one-way ANOVA by comparison with empty vector-transfected controls. ns $p>0.05$; * $p<0.05$; ** $p<0.01$. (d) Expression of CDK4 following 5 days siRNA-mediated knockdown of CDC37 (O3 and O4) in empty vector (V) or CDC37-overexpressing (C1) HCT116 cells. Controls are non-transfected (NT), mock transfected (Mk) and inactive siRNA (IC3 and IC4).

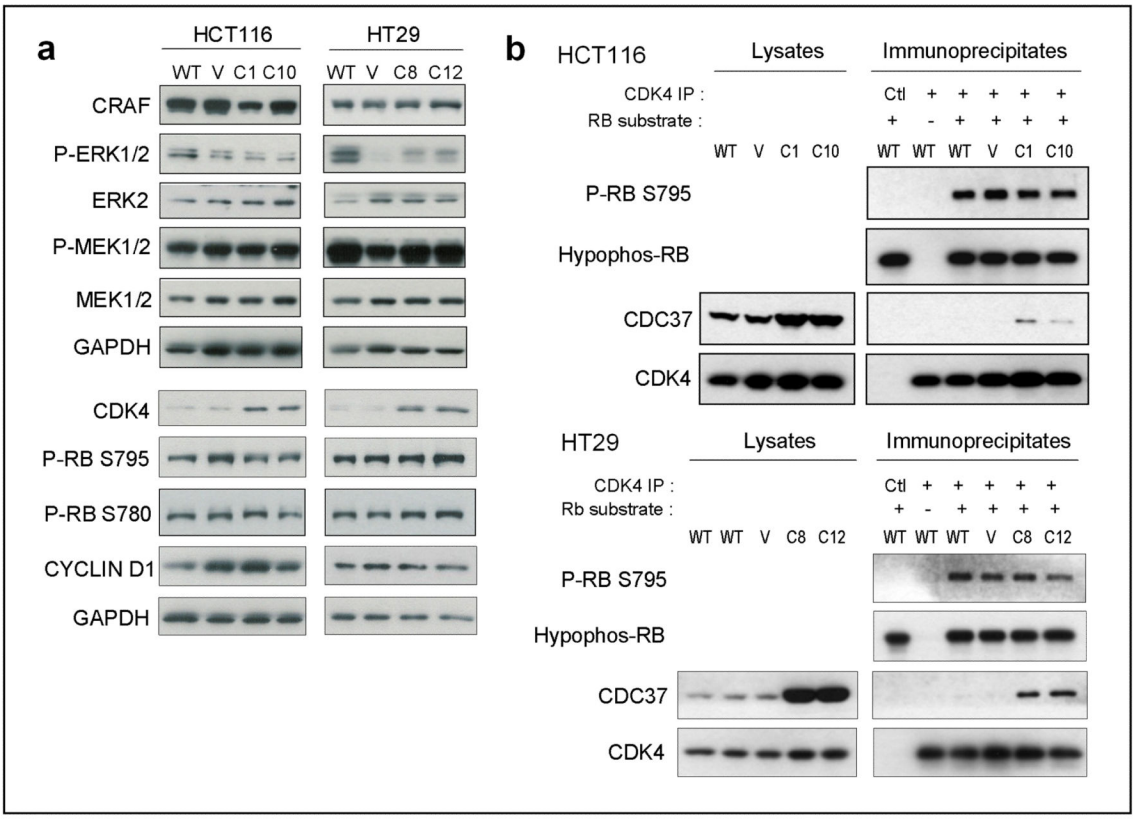


**Figure 2. Kinase client signalling activity following CDC37 overexpression**

(a) Western blot showing phosphorylated ERK1/2, ERK2, phosphorylated MEK1/2, MEK1/2, phosphorylated RB (S795 and 780) and CYCLIN D1 expression in wildtype (WT), empty vector-transfected (V) or CDC37-overexpressing HCT116 (C1, C10) and HT29 (C8, C12) cells. ERK1/2 phosphorylation is quantified by densitometry in Supplementary Figure S3. (b) CDK4 kinase assay in wildtype (WT), empty vector-transfected (V) or CDC37-overexpressing HCT116 (C1, C10) and HT29 (C8, C12) cells. Following CDK4 immunoprecipitation, and incubation with recombinant RB, S795-phosphorylated RB and hypophosphorylated RB were measured by western blotting.




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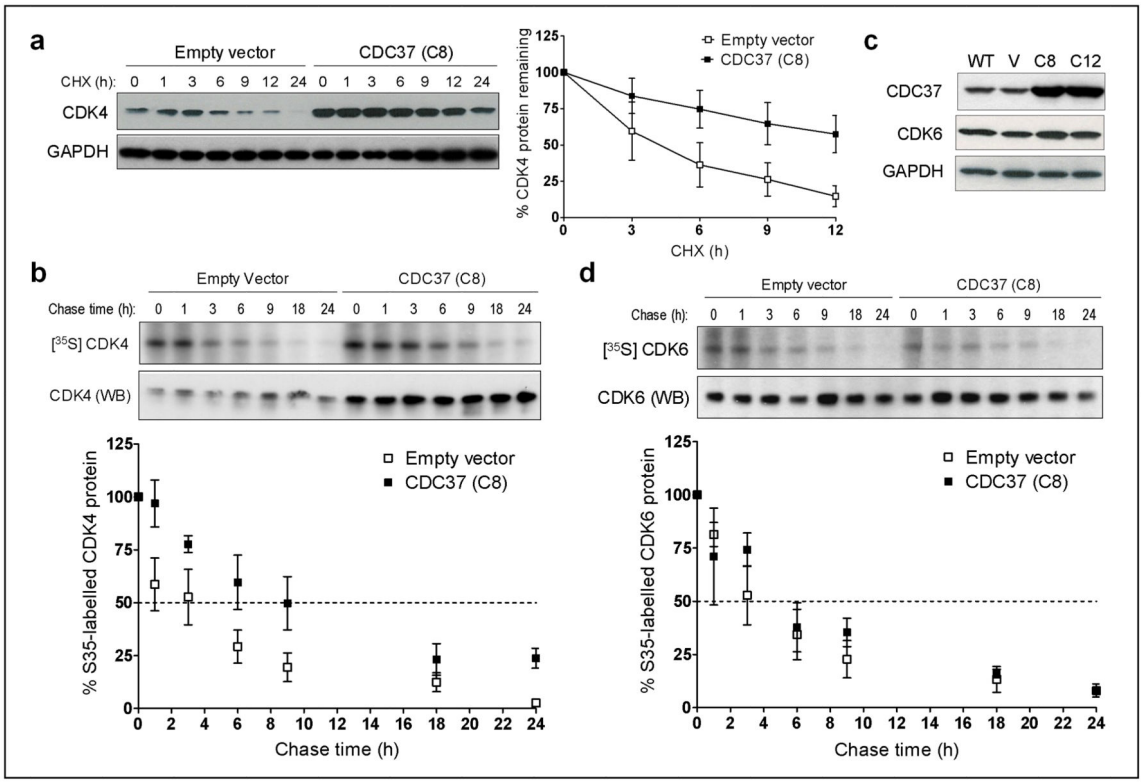


**Figure 3. Kinase client protein stability in CDC37-overexpressing cells**

(a) Time course of CDK4 expression in empty vector-transfected or CDC37-overexpressing (C8) HT29 colon cancer cells following exposure to 20μg/ml cycloheximide (CHX). Left: Western blot, right: Mean densitometry of percentage CDK4 (relative to 0h) normalised to GAPDH ±SE from 3 independent repeats. (b) Pulse chase with 2h exposure to 150μCi/ml $^{35}$S labelled methionine/cysteine and CDK4 immunoprecipitation. CDC37-overexpressing clone C8 was compared with HT29 empty vector-transfected cells. Total CDK4 levels in each immunoprecipitate were determined by western blotting (WB). Below: Mean densitometry of percentage labelled CDK4 remaining ±SE from 3 independent repeats. (c) Expression of CDK6 in wildtype (WT), empty vector-transfected (V) or CDC37-overexpressing (C8 and C12) HT29 cells. (d) Pulse chase with 2h exposure to 150μCi/ml 35S labelled methionine/cysteine and CDK6 immunoprecipitation. CDC37-overexpressing clone C8 was compared with HT29 empty vector-transfected cells. Total CDK6 levels in each immunoprecipitate were determined by western blotting (WB). Below: Mean densitometry of percentage labelled CDK6 remaining ±SE from 3 independent repeats.

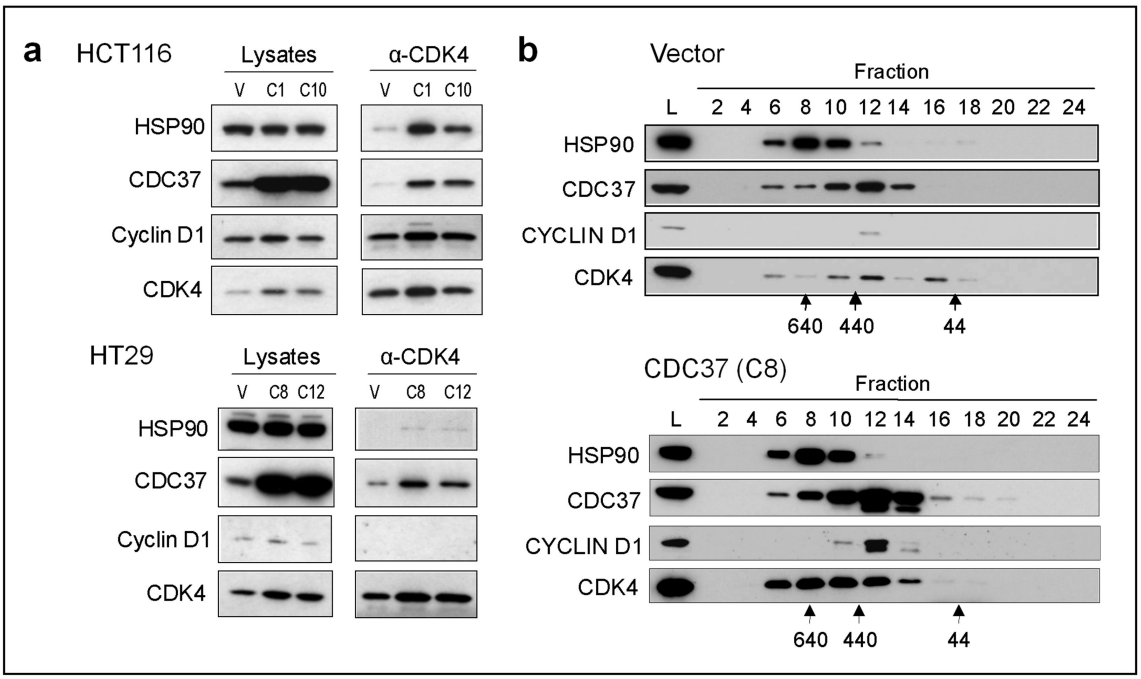


**Figure 4. CDK4 protein complexes in CDC37-overexpressing cells**

(a) Western blot for HSP90, CDC37, CYCLIN D1 and CDK4 in whole cell lysates or CDK4 immunoprecipitates from empty vector-transfected (V) or CDC37-overexpressing HCT116 (C1, C10) and HT29 (C8, C12) colon cancer cells. The levels of HSP90 coimmunoprecipitated with CDK4 in HCT116 cells are quantified by densitometry in Supplementary Figure S4a. (b) Western blot analysis of whole cell lysates (L) and fractions following Superose12 gel filtration separation of complexes. Approximate molecular weights are shown. CDC37-overexpressing clone C8 (HT29) is compared with HT29 empty vector-transfected clone.




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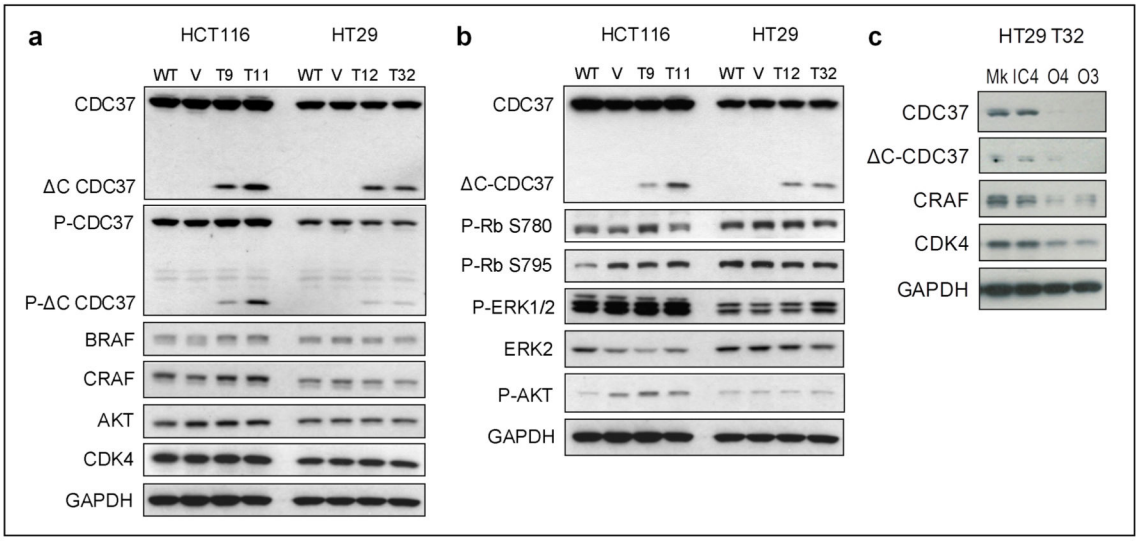


**Figure 5. Kinase client expression and signalling activity in cells expressing ΔC-CDC37**

(a) Western blot showing kinase clients and phospho-S13-CDC37 expression in wildtype (WT), empty vector-transfected (V) or ΔC-CDC37-expressing HCT116 (T9, T11) and HT29 (T12, T32) colon cancer cells. Client protein expression is quantified by densitometry in Supplementary Figure S5. (b) Kinase client signalling output as measured by western blot analysis of phosphorylated RB (S780 and S795), phosphorylated ERK1/2 and S473-phosphorylated AKT in wildtype (WT), empty vector-transfected (V) or ΔC-CDC37-expressing HCT116 (T9, T11) and HT29 (T12, T32) colon cancer cells. (c) Client protein expression following 5 days knockdown of endogenous CDC37 (O3 and O4) in ΔC-CDC37-expressing HT29 clone T32. Controls are no transfection (NT), mock transfection (Mk) and inactive siRNA (IC3 and IC4).




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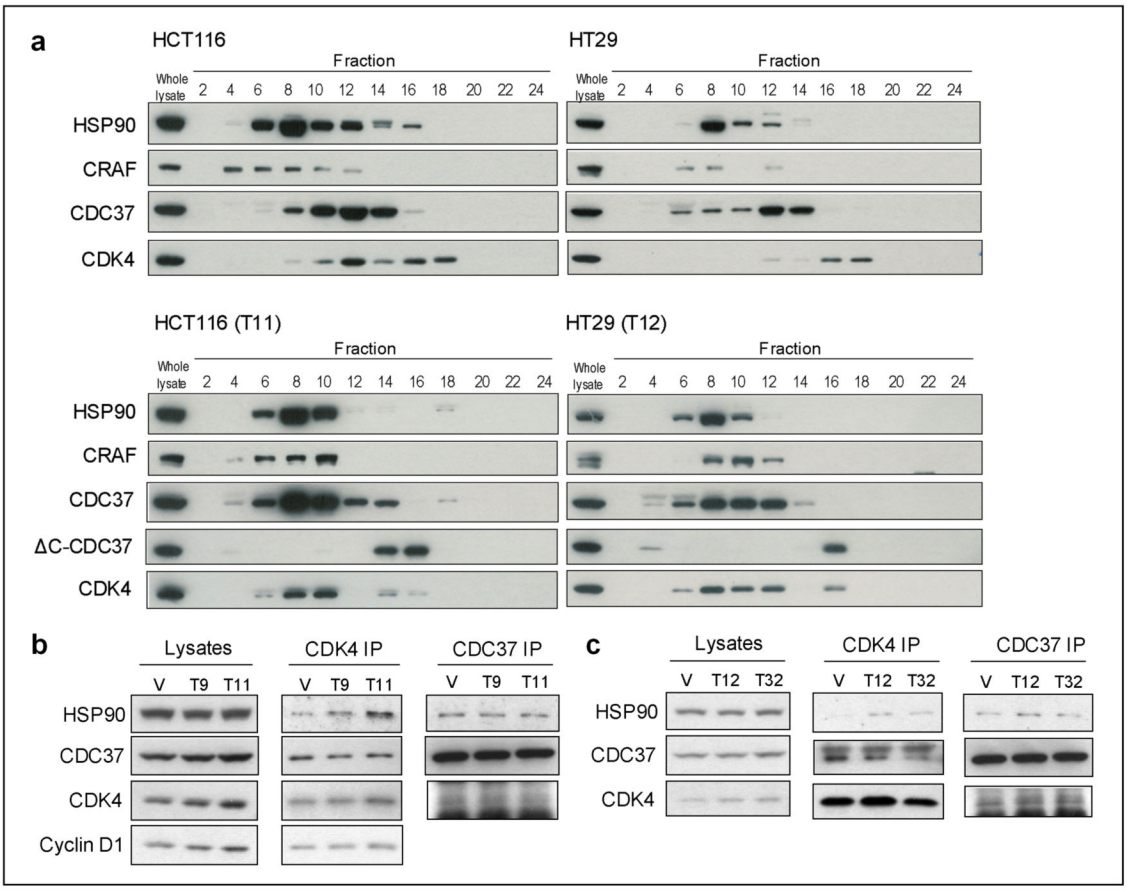


**Figure 6. Kinase client chaperone complexes in cells expressing ΔC-CDC37**

(a) Western blot analysis of whole cell lysates and fractions following gel filtration separation of complexes using a Superose12 column. Parental HCT116 and HT29 cells are compared with ΔC-CDC37-expressing clones T11 and T12, respectively. (b) & (c) Immunoprecipitates of CDK4 and CDC37 from empty vector-transfected (V) or ΔC-CDC37-expressing clones (b) HCT116 (T9, T11), and (c) HT29 (T12, T32). The levels of HSP90 co-immunoprecipitated with CDK4 in all HCT116 and HT29 clones are quantified by densitometry in Supplementary Figure S6.

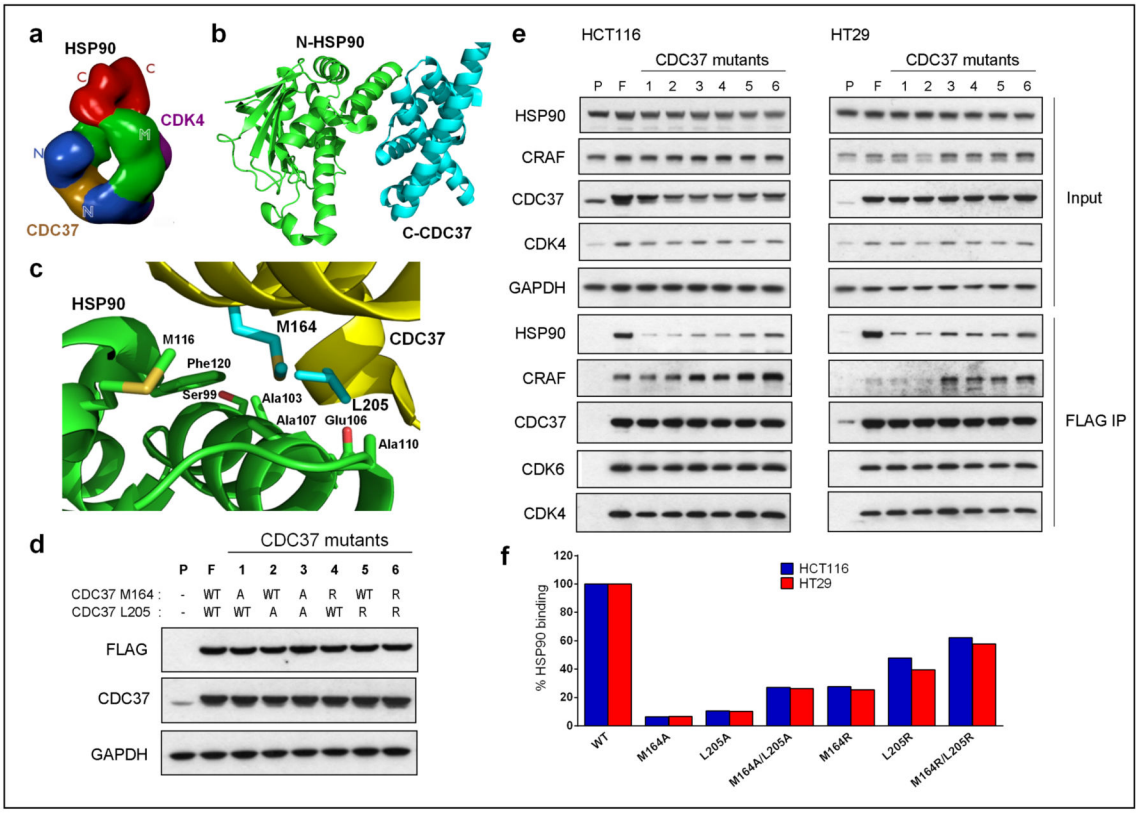


**Figure 7. CDC37 point mutations reduce binding to HSP90**

Cartoons showing interactions between HSP90 and CDC37: (a) The EM structure of the HSP90-CDC37-CDK4 complex. CDC37 (gold) interacts with the HSP90 N-terminal domains (blue). CDK4 (magenta) interacts with and the middle-domain (green) and C-terminal domain (red) of HSP90. (b) The N-terminal domain of yeast HSP90 bound to the C-terminal domain of CDC37. (c) CDC37 (yellow) residues, M164 and L205 (shown in cyan), packing against the yeast HSP90 N-terminal domain (green). While mutation of M164 to alanine can be accommodated the mutation of L205 to the bulkier arginine cannot be easily accommodated. (d) Overexpression of FLAG-CDC37 (F) or 6 different FLAG-CDC37 point mutants in HCT116 cells in comparison with the parental cells (P). The level of expression of CDC37 mutant 1 (M164A) is estimated by densitometry in Supplementary Figure S8. (e) Inputs and FLAG immunoprecipitates from HCT116 and HT29 cells overexpressing FLAG-CDC37 (F) or 6 different FLAG-CDC37 point mutants in comparison with parental cells (P). (f) Quantitation by densitometry of FLAG-CDC37 mutants binding to HSP90 relative to FLAG-CDC37. CDC37 mutants are numbered as follows 1-M164A; 2-L205A; 3-M164A/L205A; 4-M164R; 5-L205R; 6-M164R/L205R.




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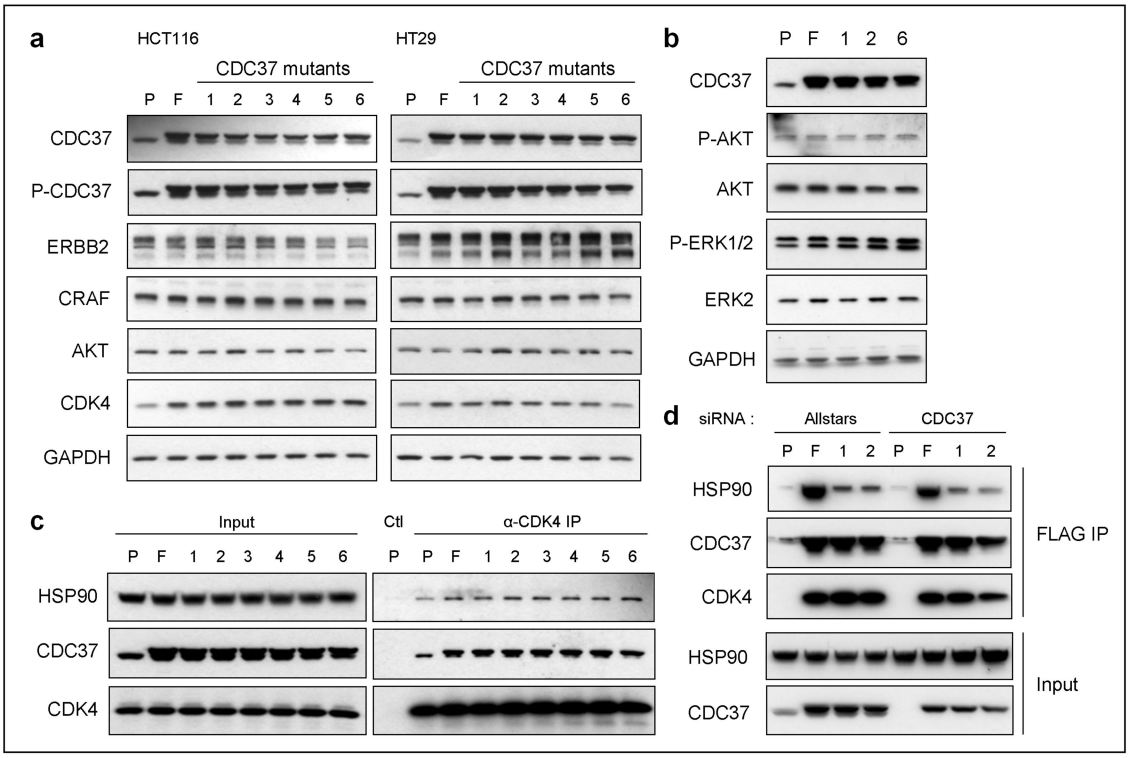


**Figure 8. Kinase client expression, signaling activity and protein complexes following expression of CDC37 mutants**

(a) Kinase client and S13-phosphorylated-CDC37 expression in HCT116 and HT29 cells. Overexpression of FLAG-CDC37 (F) or 6 different FLAG-CDC37 point mutants is compared with parental cells (P). Expression of clients CRAF and CDK4 in all cell lines is quantified by densitometry in Supplementary Figure S9. (b) S473-phosphorylated AKT and phosphorylated ERK1/2 expression in parental (P), FLAG-CDC37-overexpressing (F) or 3 different FLAG-CDC37 point mutant-overexpressing HCT116 cell clones. (c) Inputs and CDK4 immunoprecipitates from parental (P), FLAG-CDC37-overexpressing (F) or 6 different FLAG-CDC37 point mutant-overexpressing HCT116 cells. The levels of HSP90 coimmunoprecipitated with CDK4 are quantified by densitometry in Supplementary Figure S10. (d) Inputs and FLAG immunoprecipitates from parental (P), FLAG-CDC37-overexpressing (F) or 2 different FLAG-CDC37 point mutant-overexpressing HCT116 cell lines after 5 days transfection of Allstars negative control or CDC37 siRNA. CDC37 mutants are numbered as follows 1-M164A; 2-L205A; 3-M164A/L205A; 4-M164R; 5-L205R; 6-M164R/L205R.




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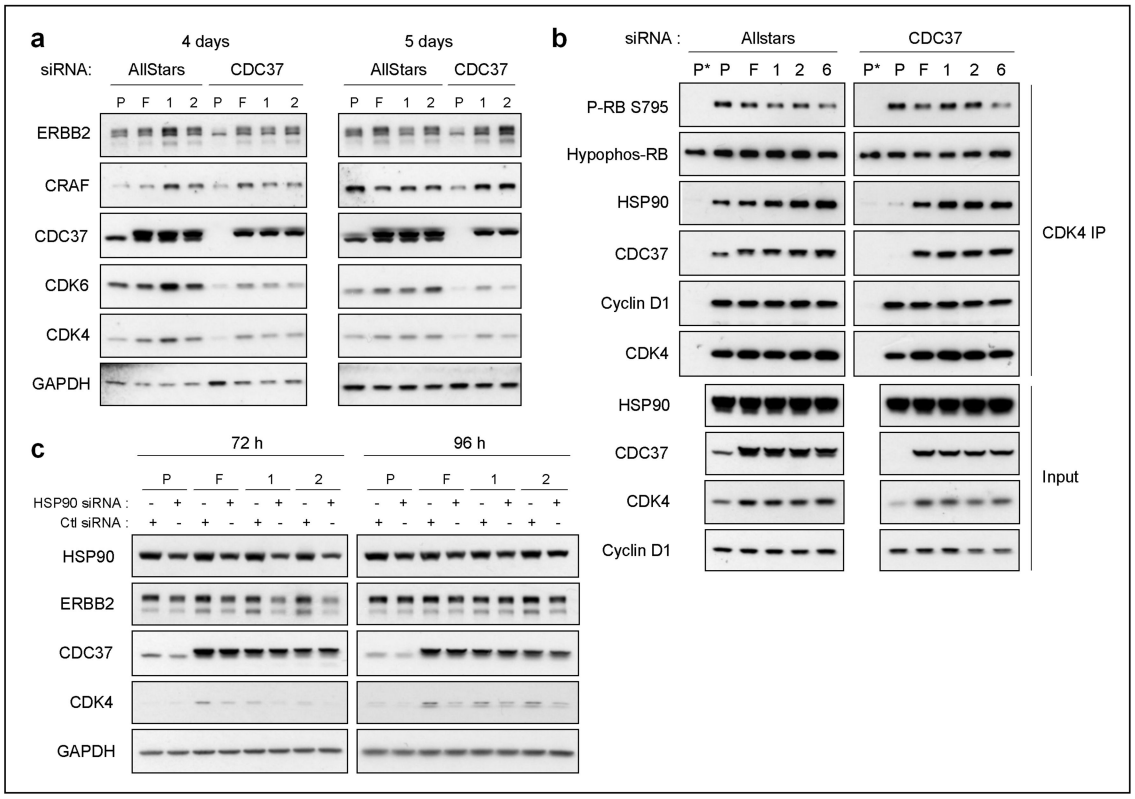


**Figure 9. Rescue of wildtype CDC37 but not HSP90 function with CDC37 mutants**

(a) Expression of kinase clients after knockdown with CDC37 siRNA or Allstars negative control siRNA for 4 or 5 days, comparing parental (P), FLAG-CDC37-overexpressing (F) and 2 different FLAG-CDC37 point mutant-overexpressing HCT116 cell lines. The rescue of CDK4, ERBB2 and CRAF client expression is quantified by densitometry in Supplementary Figure S11. (b) CDK4 kinase assay following 5 days transfection with Allstars negative control siRNA or CDC37 siRNA in parental (P), FLAG-CDC37-overexpressing (F) or 3 different FLAG-CDC37 point mutant-overexpressing HCT116 cell lines. Following CDK4 immunoprecipitation, and incubation with recombinant RB, S795-phosphorylated RB and hypophosphorylated RB were measured by western blotting. A control * without CDK4 IP is included. (c) Kinase client expression after transfection of HSP90α and HSP90β or inactive siRNA in parental (P), FLAG-CDC37-overexpressing (F) or 2 different FLAG-CDC37 mutant-overexpressing HCT116 cell lines. CDC37 mutants are numbered as follows 1-M164A; 2-L205A; 6-M164R/L205R.

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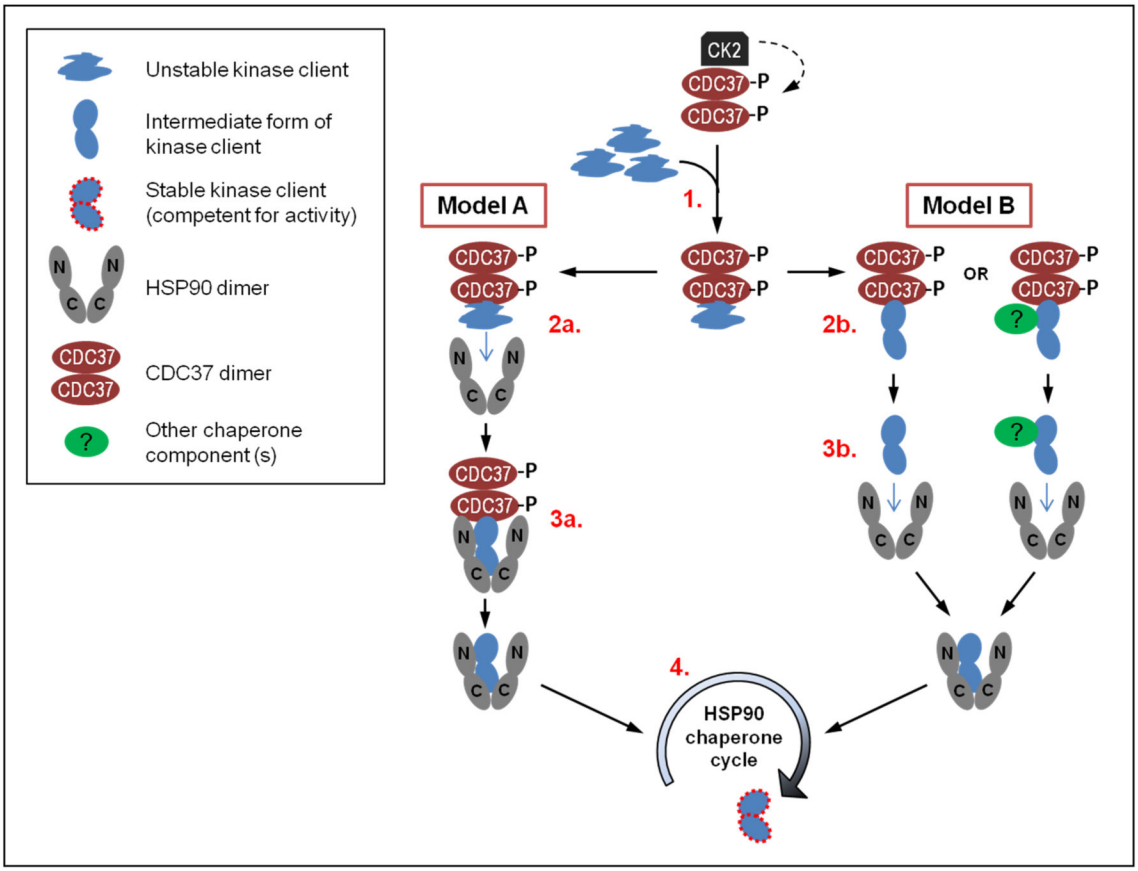


**Figure 10. Models for the mechanism of action of CDC37 in the chaperoning of kinase clients**

Two proposed models for the role of CDC37 in the chaperoning of kinase client proteins. Model A involves CDC37 directly binding to HSP90 thereby bringing kinase clients to the chaperone complex whereas Model B involves CDC37 altering kinases to promote their interaction with the chaperone complex as detailed in the numbered steps below. (1) CDC37 is activated by phosphorylation on Ser13 by CK2 and interacts with unstable protein kinase clients. According to Model A, (2a) CDC37 directly brings kinase clients to HSP90 chaperone complexes. (3a) CDC37 interacts with the N-terminus of HSP90 allowing the two lobes of the kinase client to interact in the correct conformation with HSP90 and (4) progression of the ATPase-dependent HSP90 chaperone cycle resulting in the formation of stable kinases competent for activity. In the alternative mechanism (Model B), (2b) the conformation of kinase clients bound to CDC37 is altered in such a way that may allow the two lobes of the kinase to be more amenable for interaction with HSP90. This could involve other components of the HSP90 chaperone system. (3b) The re-orientation of the kinase structure may allow the kinase to be released from CDC37 and bind to HSP90. This may or may not involve support from other proteins.

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## Table 1

**17-AAG $GI_{50}$ (nM) in HCT116 cell lines with knockdown of endogenous CDC37 or control siRNA**

| Cell line | Allstars siRNA | CDC37 siRNA |
|---|---|---|
| Parental | 49.7 ±2 | * 21.7 ±5 |
| CDC37 | 46.3 ±2 | 39.0 ±12 |
| CDC37 M164A | 47.7 ±3 | 36.0 ±3 |
| CDC37 L205A | 54.0 ±8 | 49.7 ±20 |
| CDC37 M164R/L205R | 51.7 ±4 | 45.3 ±8 |

* $p<0.05$ comparing $GI_{50}$ with CDC37 to Allstars siRNA

Mean ±SE from 3 independent experiments