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ORIGINAL ARTICLE

Suppression of Her2/*neu* expression through ILK inhibition is regulated by a pathway involving TWIST and YB-1

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In a previous study it was found that the therapeutic effects of QLT0267, a small molecule inhibitor of integrin-linked kinase (ILK), were influenced by Her2/*neu* expression. To understand how inhibition or silencing of ILK influences Her2/*neu* expression, Her2/*neu* signaling was evaluated in six Her2/*neu*-positive breast cancer cell lines (LCC6[Her2], MCF7[Her2], SKBR3, BT474, JIMT-1 and KPL-4). Treatment with QLT0267 engendered suppression (32–87%) of total Her2/*neu* protein in these cells. Suppression of Her2/*neu* was also observed following small interfering RNA-mediated silencing of ILK expression. Time course studies suggest that ILK inhibition or silencing caused transient decreases in P-AKT[ser473], which were not temporally related to Her2/*neu* downregulation. Attenuation of ILK activity or expression was, however, associated with decreases in YB-1 (Y-box binding protein-1) protein and transcript levels. YB-1 is a known transcriptional regulator of Her2/*neu* expression, and in this study it is demonstrated that inhibition of ILK activity using QLT0267 decreased YB-1 promoter activity by 50.6%. ILK inhibition was associated with changes in YB-1 localization, as reflected by localization of cytoplasmic YB-1 into stress granules. ILK inhibition also suppressed TWIST (a regulator of YB-1 expression) protein expression. To confirm the role of ILK on YB-1 and TWIST, cells were engineered to overexpress ILK. This was associated with a fourfold increase in the level of YB-1 in the nucleus, and a 2- and 1.5-fold increase in TWIST and Her2/*neu* protein levels, respectively. Taken together, these data indicate that ILK regulates the expression of Her2/*neu* through TWIST and YB-1, lending support to the use of ILK inhibitors in the treatment of aggressive Her2/*neu*-positive tumors.
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Introduction

Increased integrin-linked kinase (ILK) expression and/ or activity (Graff *et al.*, 2001; Bravou *et al.*, 2003; Obara *et al.*, 2004; Takanami, 2005; Sawai *et al.*, 2006) has been documented in many cancers types, including lung (Takanami, 2005), brain (Obara *et al.*, 2004), prostate (Graff *et al.*, 2001), pancreatic (Sawai *et al.*, 2006), colon (Bravou *et al.*, 2003, 2006), gastric (Ito *et al.*, 2003) and ovarian (Ahmed *et al.*, 2003) cancers and malignant melanomas (Dai *et al.*, 2003). Overexpression of ILK in epithelial cells has been shown to induce epithelial–mesenchymal transition (Li *et al.*, 2003, 2007; Oloumi *et al.*, 2004, 2006) and deregulated growth, whereas targeted inhibition of ILK induces apoptosis and cell cycle arrest (Persad *et al.*, 2000; Persad and Dedhar, 2003; Duxbury *et al.*, 2005; McDonald *et al.*, 2008a). In normal mammary cells, overexpression of ILK stimulates anchorage-independent cell growth (Hannigan *et al.*, 1997; Radeva *et al.*, 1997; Kumar *et al.*, 2004), and causes constitutive upregulation of cyclin D and A expression while promoting cell cycle progression (Radeva *et al.*, 1997), hyperplasia and tumor formation *in vivo* (White *et al.*, 2001). Given the importance of ILK in cancer development and progression, it is anticipated that ILK inhibition and/or silencing may be an effective way of treating cancer. Preclinical studies completed to date support this idea (Edwards *et al.*, 2008; Kalra *et al.*, 2009).

A recent study from our lab using preclinical breast cancer models highlighted the therapeutic benefits associated with targeting ILK (Kalra *et al.*, 2009). However, the results clearly indicated that Her2/*neu*-positive breast cancer cell lines responded uniquely when compared with cell lines that expressed low levels of Her2/*neu*. For example, Her2/*neu*-positive tumors were more sensitive to treatment with QLT0267. The

studies summarized here investigated Her2/*neu* expression in six cell lines where Her2/*neu* overexpression was a result of gene amplification (SKBR3, BT474, JIMT-1 and KPL-4) or gene transfection (LCC6[Her2] MCF7[Her2]). The results presented demonstrate that ILK inhibition (with a small molecule ILK inhibitor, QLT0267) or silencing (using small interfering RNA (siRNA)) suppressed Her2/*neu* protein expression. Evidence is provided to suggest that ILK-mediated regulation of Her2/*neu* appears to act through signaling pathways involving the transcription factors Y-box binding protein-1 (YB-1) and TWIST.

Results

QLT0267 or ILK-targeted siRNA suppress total Her2/neu expression in multiple breast cancer cell lines

In an effort to better understand the effects of QLT0267 on Her2/*neu*-positive breast cancer cells, the expression of total Her2/*neu* was examined in cell lines that were treated with QLT0267 at various doses for a 24 h time point that was selected based on Alamar Blue assay (Medicorp Inc., Montreal, QC, Canada) that demonstrate no decreases in cell viability at this time (Figure 1). All six breast cancer cell lines examined, including LCC6[Her2] (Figure 1a), MCF7[Her2] (Figure 1b), BT474 (Figure 1c), KPL4 (Figure 1d), SKBR3 (Figure 1e) and JIMT-1 (Figure 1f), showed a reduction in total Her2/*neu* protein levels in response to exposure to QLT0267. Her2/*neu* levels in cells treated with QLT0267 were qualitatively assessed by densitometry (average of three independent experiments) and the results indicated that in all cell lines 42 μm QLT0267 resulted in suppression of total Her2/*neu*. Levels were decreased by 69, 86.5, 49, 47, 63 and 32% in LCC6[Her2] MCF7[Her2], BT474, KPL4, SKBR3 and JIMT-1 cells, respectively. To understand why LCC6[Her] cells showed significant downregulation of Her2/*neu* at a concentration up to fourfold lower than the other cell lines tested, we performed reverse transcriptase–PCR to compare the level of Her2/*neu* mRNA in SKBR3 cells relative to LCC6[Her2] cells. The analysis showed that SKBR3 cells have 48-fold more Her2/*neu* transcript than the LCC6[Her] cell line.

To determine if the suppression of Her2/*neu* was a direct or indirect effect of QLT0267, SKBR3 were transiently nucleofected with 2 μg ILK siRNA or a universal siRNA control (Neg) and ILK, AKT P-AKT[ser473] and Her-2/*neu* levels were determined at 24, 48, 72 and 96 h (see representative blots in Figure 2). ILK expression was decreased by an average of 49, 66, 66 and 79% at 24, 48, 72 and 96 h, respectively. Total Her2/*neu* expression was decreased by 71% at 96 h (Figure 2a).

An analysis of phosphorylation of AKT at serine 473 was done to elucidate whether the mechanism through which ILK modulates the expression of Her2/*neu* involves its downstream target, AKT. The results demonstrate that ILK silencing is associated with significant decreases in P-AKT[ser473] levels, but the effect is transient.
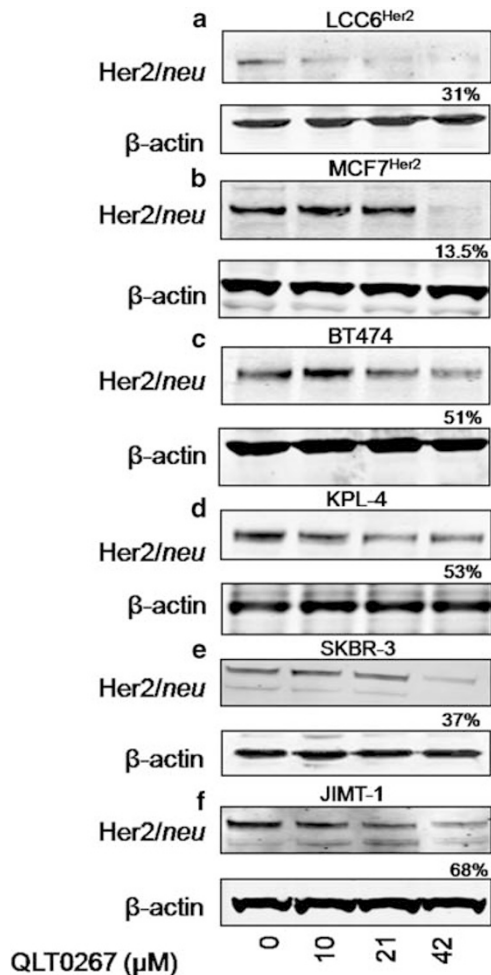

Figure 1 Her2/*neu* expression following treatment of various breast cancer cell lines with QLT0267. Expression of total Her2/*neu* in (**a**) LCC6[Her2], (**b**) MCF7[Her2], (**c**) BT474, (**d**) KPL4, (**e**) SKBR3 and (**f**) JIMT-1 cells treated with QLT0267 was determined using western blot analysis. Cells were treated for 24 h with 10, 21 or 42 μM QLT0267. Subsequently, cells were lysed, proteins were isolated and 50 μg whole-cell lysates were separated on 10% SDS–PAGE gels as described in the Materials and methods. Membranes were probed for Her2/*neu* and β-actin. In all six cell lines, increasing concentrations of QLT0267 inhibited the expression of total Her2/*neu*. At 42 μm, total Her2/*neu* is decreased by 69, 86.5, 49, 47, 63, and 32% ($n = 3$) in LCC6[Her2], MCF7[Her2], BT474, KPL4, SKBR3 and JIMT-1 cells, respectively.

Within 24 h of treatment using ILK-targeted siRNA, there was 79% suppression of P-AKT[ser473]. These values returned to control levels by 72 h (Figure 2a). P-AKT[ser473] levels in SKBR3 cells were also determined following treatment with QLT0267 (Figure 2b). Significant decreases in P-AKT[ser473] were observed at 6 and 18 h; however, P-AKT[ser473] levels began to increase by 24 h (Figure 2b). Similar results were seen in the LCC6[Her2] cell line. Transient decreases in P-AKT[ser473] levels following inhibition or silencing of ILK is consistent with the initiation of compensation mechanisms as reported by others (Troussard *et al.*, 2006; McDonald *et al.*, 2008a). Interestingly, although JIMT-1 cells experience a decrease in Her2/*neu* levels after
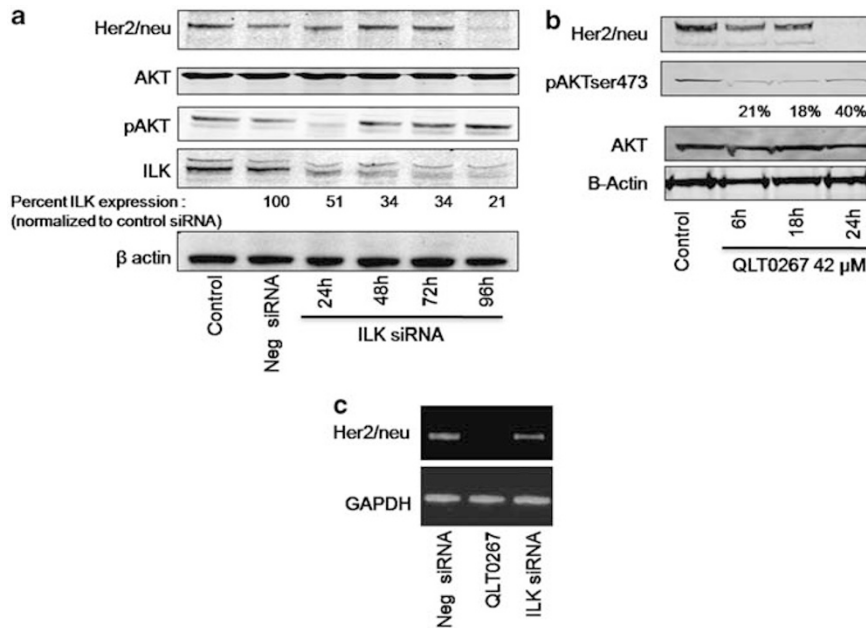
Figure 2 (**a**) Pathway analysis of SKBR3 cells transiently nucleofected with 2 μg of ILK siRNA using the Amaxa Nucleofector. Whole-cell lysates (50 μg) harvested from cells at 24, 48, 72 and 96 h post transfection were separated on 10% SDS–PAGE gels. Resulting western blots were probed for ILK, Her2/*neu*, AKT, PAKT^{ser473} and β-actin to verify loading. ILK expression was decreased by 49, 66, 66 and 79% at 24, 48, 72 and 96 h, respectively. PAKT^{ser473} was suppressed by 79% at 24 h where ILK silencing was at 49%. At 48 h of treatment with ILK siRNA, SKBR3 cells exhibit a 66% suppression of ILK. At this and later time points, PAKT^{ser473} expression is similar to control cells. Total Her2/*neu* expression was reduced by 71% at 96 h of treatment with ILK siRNA when compared with the Neg siRNA (*n* = 3). (**b**) SKBR3 cells were treated with 42 μM QLT0267 for 6, 18 or 24 h. Subsequently, cells were lysed, 50 μg of protein was isolated and then separated on 10% SDS–PAGE gels. Resulting western blots were probed for Her2/*neu*, PAKT^{ser473} and β-actin to verify loading. Treatment with QLT0267 suppressed PAKT^{ser473} in all cell lines at a time point earlier than that observed to suppress Her2/*neu*. PAKT^{ser473} was decreased at 6 h, whereas Her2/*neu* levels decreased substantially at 24 h, where PAKT^{ser473} begins to increase. (**c**) SKBR3 cells were treated with 42 μM QLT0267 for 24 h or transfected with ILK siRNA. Subsequently, RNA was isolated from cells and reverse transcribed. Her2/*neu* was amplified from complementary DNA (cDNA) using quantitative reverse transcriptase–PCR (RT–qPCR) and PCR. A 9.8- and 2.5-fold decrease of Her2/*neu* transcript was observed when cells were treated using QLT0267 or ILK siRNA.

treatment with 42 μM of QLT0267, these cells do not show decreased P-AKT at any of the time points tested (data not shown).

In order to determine whether ILK silencing by siRNA or inhibition by QLT0267 affected Her2/*neu* transcription, RNA was isolated from SKBR3 cells treated with QLT0267 or transfected with ILK siRNA. Her2/*neu* mRNA was measured using PCR and the results, based on three independent experiments, indicated that inhibition (QLT0267) or silencing (siRNA) of ILK was associated with 9.8- and 2.5-fold decreases in Her2/*neu* transcript levels, respectively (Figure 2c).

Influence of ILK inhibition or silencing on YB-1 expression and intracellular localization
ILK silencing/suppression decreased Her2/*neu* expression in both SKBR3 cells, where overexpression is because of c-*erbB2* gene amplification, and in LCC6^{Her2} cells, where Her2/*neu* expression is the result of c-erbB2 gene transfection driven by the RSV-LTR (long terminal repeat of Rous sarcoma virus) promoter; thus, in our minds, the potential mechanisms through which ILK modulates Her2/*neu* were not limited to transcriptional control. Rather, mechanisms that could influence

Her2/*neu* expression in these different cell types might therefore involve transcription factors such as activator protein-2 and *Pseudomonas* exotoxin A, stabilization factors such as heat shock proteins 70 and 90 and translational mechanisms such as YB-1. There was a strong rationale for examining whether ILK regulates YB-1 expression and/or cellular localization, as both could trigger changes in Her2/*neu* expression (Kohno *et al*., 2003; Bergmann *et al*., 2005; Berquin *et al*., 2005; Kedersha and Anderson, 2007; Lo *et al*., 2007; Takemoto *et al*., 2009; Chernov *et al*., 2008a, b, 2009; Evdokimova *et al*., 2006a, b; Pontier *et al*., 2010). SKBR3 cells were transiently nucleofected with ILK siRNA. Subsequently, ILK, Her2/*neu*, total YB-1 and β-actin levels were determined by western blot analysis. The results, summarized in Figure 3a, indicate that ILK levels were 64 and 84% suppressed at the 24 and 48 h time points, respectively. Consistent with the results summarized in Figure 2a, ILK silencing was associated with significant decreases in total Her2/*neu* levels. These studies also demonstrated that ILK silencing was associated with decreases in YB-1 expression, where 48 h after ILK siRNA addition, YB-1 protein levels were reduced by 74%. Similar results were seen in LCC6^{Her2} cell lines (Supplementary Figure 2A). Further-
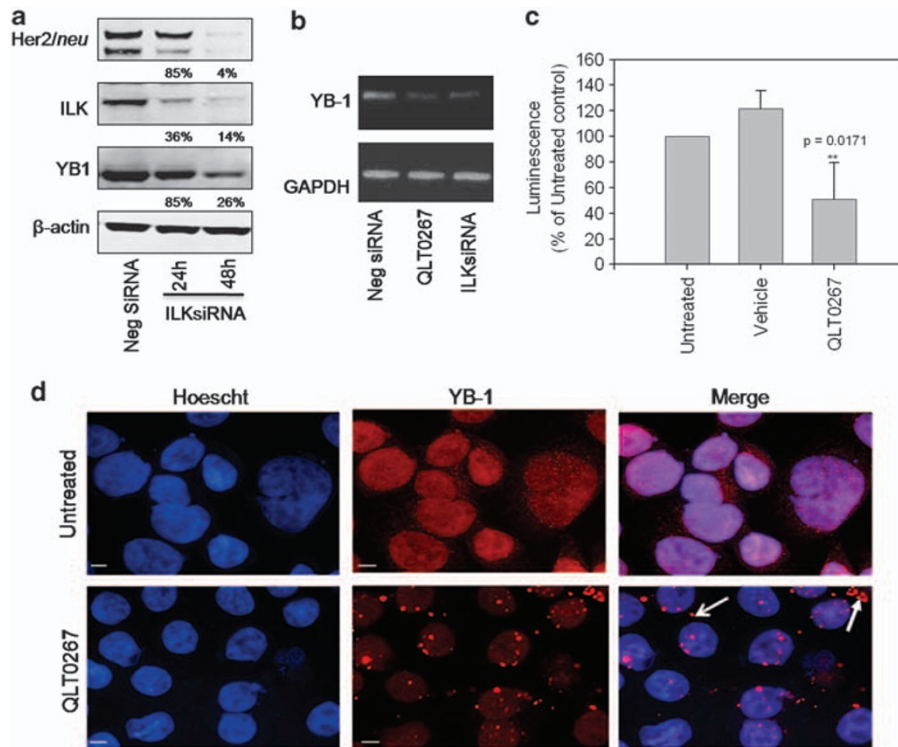
Figure 3 Inhibition of ILK activity or expression influences YB-1 transcription and subcellular localization. (**a**) SKBR3 cells were transiently nucleofected with 4 µg ILK siRNA. Subsequently, cells were lysed and 50 µg of protein was isolated from samples at 24 and 48 h, separated on a 10% SDS–PAGE gel and probed for ILK, Her2/neu, YB-1 and β-actin to verify loading. ILK expression was substantially silenced when SKBR3 cells were treated with 4 µg of ILK siRNA for both 24 and 48 h. Cells exhibit a 96% decrease in total Her2/neu expression after 48 h, at which time YB-1 expression is reduced by 74%. (**b**) YB-1 transcript levels were analyzed in SKBR3 cells treated with QLT0267 or nucleofected with 4 µg ILK siRNA for 48 h using PCR. A 9.9-fold and 6.5-fold decrease in YB-1 transcript was observed in QLT0267-treated and ILK-silenced cells, respectively, when compared with control. (**c**) SKBR3 cells were transfected with a YB-1 promoter/luciferase construct and treated with QLT0267 or vehicle control (PTE) for 24 h. A significant reduction in YB-1 promoter activity of 50% is achieved when cells are treated with QLT0267 when compared with untreated controls ($P < 0.05$) (**d**) SKBR3 cells grown on coverslips were treated with 42 µM QLT0267 for 24 h, fixed with 4% paraformaldehyde (PFA) and then stained for YB-1. Immunofluorescent images show that treatment of SKBR3 cells trigger a decrease in YB-1 protein (red) as well as a change in localization to granular structures in the cytoplasm (white arrows). Hoechst staining was used to counter stain nuclei (blue). Bar, 5 µm.

more, ILK silencing (siRNA) and inhibition (QLT0267) engendered a 9.9- and 6.8-fold decrease in YB-1 transcript levels, respectively (Figure 3b). Similar results were seen in LCC6^Her2 cell lines (Supplementary Figure 2B).

To determine whether ILK inhibition can influence transcription of YB-1, the activity of the YB-1 promoter region was evaluated using dual luciferase reporter assay (Stratford et al., 2007, 2008). SKBR3 cells were transfected with a YB-1/luciferase construct and a thymidine kinase promoter/Renilla construct (control). Cells were treated with QLT0267 or vehicle control and changes in luciferase activity were determined (Figure 3c). The results demonstrate that QLT0267-treated cells exhibited a 50% decrease ($P < 0.05$) in YB-1 promoter activity when compared with untreated controls and normalized to results found using the control thymidine kinase/Renilla construct. It is not clear why, but the vehicle-treated cells exhibited an increase in promoter activity (Figure 3c).

To confirm the western blot data summarized in Figure 3a, immunofluorescence imaging of YB-1 was examined in SKBR3 cells treated with QLT0267.

Representative images of untreated SKBR3 cells compared with cells treated with QLT0267 are shown in Figure 3d. QLT0267-treated cells exhibited lower levels of immunofluorescence, consistent with the western blot data. In addition, the fluorescence imaging clearly demonstrated localization of YB-1 into well-defined puncta (Figure 3d, white arrows). Similar results were seen in other cell lines including LCC6^Her2 cells (Supplementary Figure 2C).

Influence of ILK overexpression on YB-1 and Her2/neu levels
To assess how ILK influences the expression of YB-1 and Her2/neu, SKBR3 cells were stably transfected with the *wild-type ILK* (*ILKWT*) gene as described in the Materials and methods. Exogenous expression of ILK in SKBR3^ILKWT cells (Figure 4a) caused a small, but reproducible, decrease in native ILK expression. In order to assess whether there were changes in YB-1 levels or distribution, the ILKWT-transfected cells were plated on coverslips fixed and
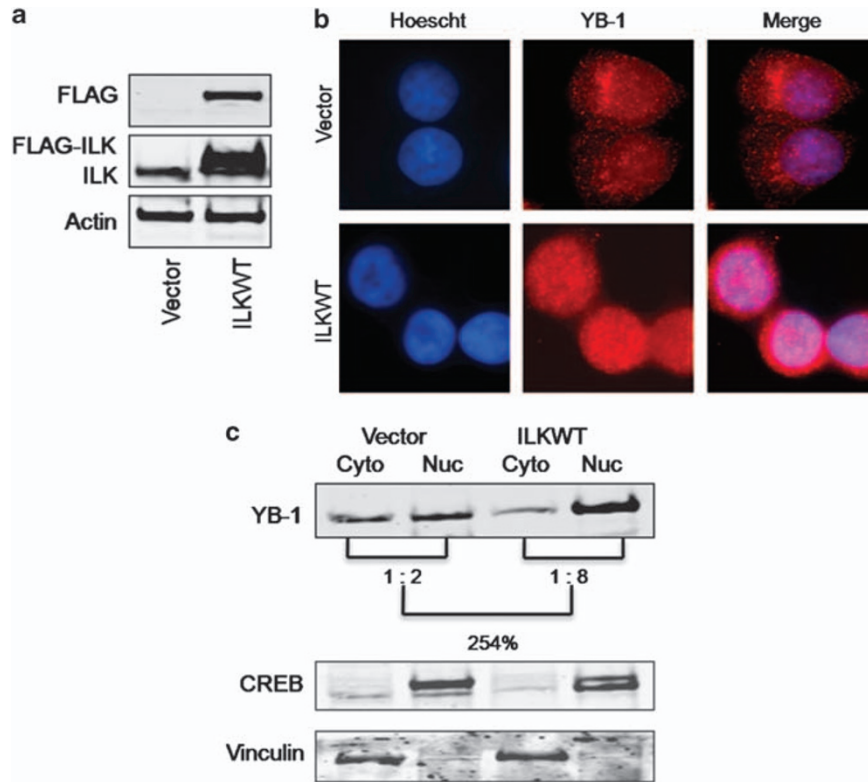
Figure 4 Overexpression of ILK in SKBR3 cells increases YB-1 expression and nuclear localization. (**a**) SKBR3 cells were stably nucleofected with an empty pIRES-hrGFP II vector or one containing a FLAG-tagged ILK gene. Cells overexpressing ILKWT or the empty vector were analyzed for ILK, FLAG and β-actin protein expression in whole-cell lysates using western blot analysis. Cells transfected with ILK exhibited double bands when blots were probed for ILK indicating the endogenous protein (lower band) and the FLAG-tagged exogenous protein (upper band). FLAG protein was readily detected in ILK-transfected cells. (**b**) SKBR3^vector and SKBR3^ILKWT cells were grown on coverslips, fixed, and then stained for YB-1 (red) and the nucleus (blue). SKBR3^vector cells exhibit a diffuse and mainly punctuate cytoplasmic pattern of YB-1 staining whereas SKBR3^ILKWT cells exhibit a mainly nuclear or peri-nuclear localization of YB-1. (**c**) A total of 50 μg of cytoplasmic and nuclear protein lysate fractions harvested from SKBR3^vector and SKBR3^ILKWT cells were separated on SDS–PAGE gels, transferred to nitrocellulose membranes and probed with anti-YB-1, and anti-vinculin and CREB to verify purity of cytoplasmic and nuclear fractions, respectively. The resulting western analysis showed that YB-1 increased by fourfold in the nuclear fraction and by 254% when considering cytoplasmic and nuclear fractions together in cells transfected with ILK when compared with vector-transfected cells.

stained for YB-1 (red) and nuclei (blue) (Figure 4b). When comparing SKBR3^ILK cells with SKBR3^vector cells, SKBR3^ILK cells exhibited increased levels of YB-1 (Figure 4b). To confirm immunofluorescence results, and to determine whether the subcellular distribution of YB-1 was changed, cytoplasmic and nuclear protein fractions (see Materials and methods) prepared from the SKBR3^vector and SKBR3^ILK cells were used in western blot analysis. As shown in Figure 4c, representative western blot analysis of YB-1 in SKBR3 cells transfected with ILK showed a 254% increase (compared with vector-transfected cells) in YB-1 when considering protein levels in the nucleus and cytoplasmic fractions. CREB (marker for the nuclear fraction) and Vinculin (marker for the cytoplasmic fraction) were used to verify the purity of the fractions (Figure 4c). The amount of YB-1 in the nucleus increased fourfold when comparing cells transfected with ILK with cells transfected with the vector. It should be noted that similar studies were completed in MCF7 cells. Forced overexpression of ILK in MCF7 cells, which express basal levels of Her2/*neu*, did not influence total YB-1 or Her2/*neu* protein levels.

However, increases in YB-1 and Her2/*neu* transcript were observed (Supplementary Figure 3).

The role of TWIST and STAT-3 in regulating YB-1 and Her2/neu expression

Changes in YB-1 levels and localization following ILK inhibition/silencing or forced overexpression provide an explanation for how ILK expression may influence Her2/*neu* expression in cell lines that overexpress Her2/*neu* because of gene amplification or transfection. The results suggest that if ILK is inhibited or suppressed, there will be a decrease in YB-1 mRNA and protein levels. It is not clear, however, how ILK would regulate the expression of YB-1, and for this reason studies were initiated to assess how ILK expression/inhibition influenced expression of the transcription factor TWIST. This protein is known to bind to the E-box regions within the YB-1 promoter and thus regulate YB-1 expression (Shiota *et al.*, 2008a, b, 2009). SKBR3 cells were treated with QLT0267 or transfected with ILK siRNA and the resulting cell lysates were blotted and
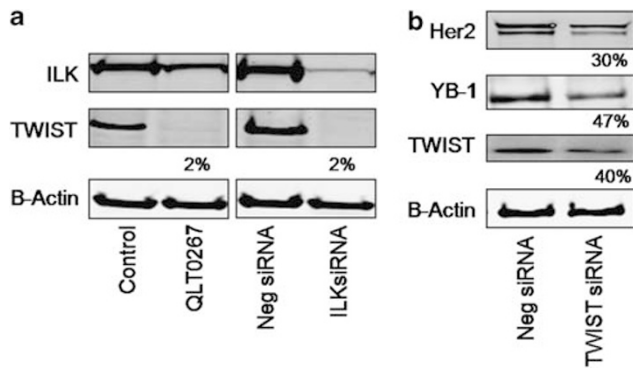
a

b

Figure 5 Inhibition of ILK activity or expression regulates TWIST expression. (**a**) SKBR3 cells were treated with 42 μM QLT0267, Neg siRNA or ILK siRNA. Subsequently, cells were lysed, protein was isolated and then separated on a 10% SDS–PAGE gel. Resulting western blots were probed for ILK, TWIST and β-actin. TWIST protein is reduced by 98% in SKBR3 cells treated with QLT0267 or nucleofected with ILK siRNA when compared with controls (untreated or Neg siRNA, respectively). (**b**) SKBR3 cells were transiently nucleofected with Control or 4 μg TWIST siRNA for 96 h. Subsequently, cells were lysed, protein was isolated from samples, separated on a 10% SDS–PAGE gel and probed for Her2/*neu*, YB-1, TWIST and β-actin. Silencing of TWIST is seen at 96 h. With a 40% silencing of TWIST, YB-1 is decreased by 47% and Her2/*neu* total protein is reduced by 70%.

probed for ILK, TWIST and β-actin. The results have been summarized in Figure 5. Treatment with QLT0267 or transfection with ILK-targeted siRNA both abrogated the expression of TWIST by 98% (Figure 5a). Similar results were seen in the LCC6[Her2] cell line (Supplementary Figure 2A). To elucidate whether a direct relationship exists between TWIST, YB-1 and Her2/*neu* expression in SKBR3 cells, the cells were nucleofected with siRNA targeting TWIST. Subsequently, the levels of TWIST, Her2/*neu*, YB-1 and β-actin were determined. Silencing TWIST proved to be quite troublesome in that several siRNA species, both custom and commercial, exhibited only minimal silencing effects. However, the results, represented by the western blot provided in Figure 5b, indicated that a 40% silencing of TWIST decreased YB-1 expression by 47% and Her2/*neu* expression by 70%. Under conditions where the SKBR3 cells were forced to overexpress ILK, TWIST expression increased by 1.9-fold (Figure 6a). Immunofluorescence analysis of TWIST in SKBR3 and SKBR3[ILKWT] was completed (Figure 6b) and these immunofluorescence studies indicated that ILK overexpression was associated with an increase in TWIST expression in the SKBR3 cells.

A key regulator of TWIST transcription is signal transducer and activator of transcription 3 (STAT-3; Ling and Arlinghaus, 2005; Lo *et al*., 2007; Cheng *et al*., 2008b). STAT-3 is activated by phosphorylation and thereafter translocates to the nucleus (Takemoto *et al*., 2009). To determine if increases in TWIST expression were the result of increased phosphorylation of STAT-3 in the ILK overexpressing SKBR3 cells, the expression of P-STAT[ser705] in the nuclear fraction of these cells was examined, and the results have been summarized in Figure 6c. Western blots probed for P-STAT[ser705],

STAT-3, CREB (nuclear marker) and vinculin (cytoplasmic marker) indicated that SKBR3 cells transfected with ILK exhibited a 225% increase (relative to the vector-transfected cells) in STAT-3 levels when considering the nuclear and cytoplasmic fractions together. Furthermore, a twofold increase in P-STAT[ser705] is observed in the nuclear fraction of SKBR3[ILKWT] cells (Figure 6c).

Discussion

It has recently been shown that the activity of QLT0267, whether used alone or in combination, was dependent on whether the breast cancer cell lines used expressed Her2/*neu* (Kalra *et al*., 2009). It was therefore important to gain a better understanding of how ILK inhibition influenced Her2/*neu* signaling and the studies described here were undertaken to address this issue. Our results demonstrate for the first time that ILK inhibition causes significant decreases in Her2/*neu* expression and that this is regulated through a previously unrecognized mechanism involving the transcription factors YB-1 and TWIST-1. To date, no direct relationship between ILK expression and Her2/*neu* signaling has been documented; however, very recently, Pontier *et al*. (2010) showed that decreased tumor induction was observed with disruption of ILK in Her2/*neu*-positive mammary epithelial tissue. Furthermore, silencing of ILK in Her2/*neu*-positive cells *in vitro* was able to block invasion and induce apoptosis (Pontier *et al*., 2010), indicating that ILK can at the very least modulate the effects of Her2/*neu* signaling.

It is clear from the results in this report that ILK-targeted siRNA or inhibition with QLT0267 engenders significant decreases in total Her2/*neu* protein levels (Figures 1 and 2). Initially, a clue to the mechanism governing this effect was identified because suppression of Her2/*neu* (because of ILK silencing/inhibition) was observed in cell lines transfected with the c-*erbB2* gene as well as in cells that overexpress Her2/*neu* because of c-*erbB2* gene amplification. Thus, it was first thought that the regulation of Her2/*neu* via ILK would involve a factor that would act at a translational level. YB-1 was previously identified as an important transcription/translation factor that participates in the formation of messenger ribonucleoprotein complexes (mRNPs; Kedersha and Anderson, 2007; Chernov *et al*., 2008a, b, 2009) and in the regulation of mRNA translation and degradation (Kohno *et al*., 2003; Evdokimova *et al*., 2006a, b). YB-1 is an oncogene that is overexpressed in a variety of cancers and its forced expression induces the development of breast cancers (Bergmann *et al*., 2005; Berquin *et al*., 2005). Previous studies indicate that normally about 90% of YB-1 protein is localized in the cytoplasm and when YB-1 is phosphorylated on serine 102, by AKT or RSK, the protein translocates to the nucleus (Sutherland *et al*., 2005; Basaki *et al*., 2007; Stratford *et al*., 2008). Nuclear localization of YB-1 is associated with increased expression of Her2/*neu* and epidermal growth factor receptor (EGFR; Wu *et al*.,
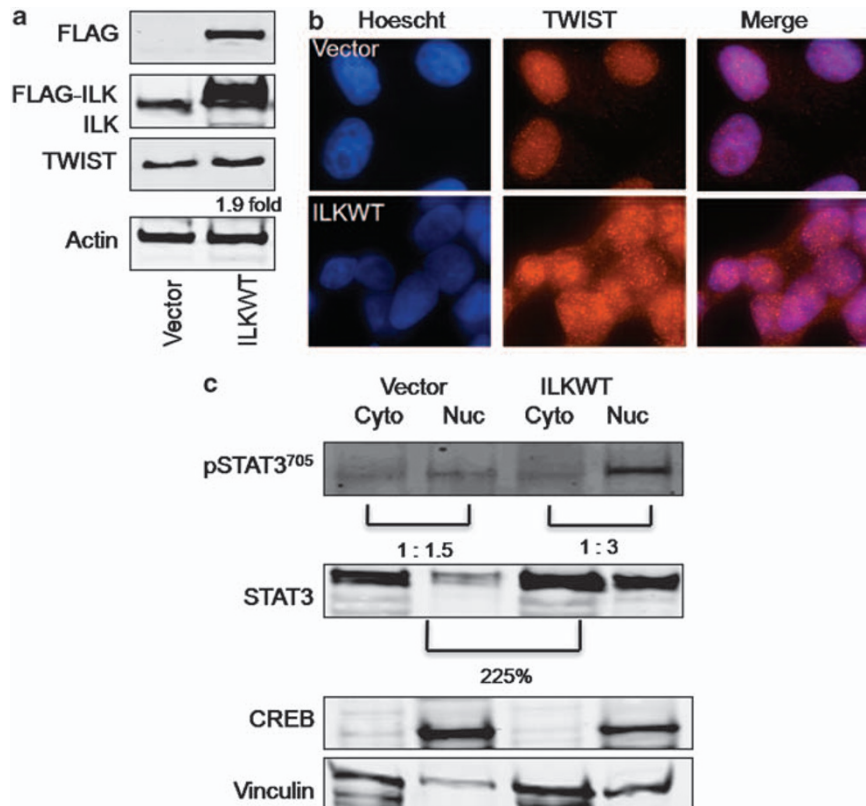
Figure 6 Overexpression of ILK increases TWIST expression through activation of STAT-3. (**a**) SKBR3 cells were stably nucleofected with an empty pIRES-hrGFP II vector or one containing a FLAG-tagged ILK gene. Cells overexpressing ILKWT or the empty vector were analyzed for ILK, TWIST and β-actin protein expression in whole-cell lysates using western blot analysis. Cells transfected with ILK exhibited double bands when blots were probed for ILK, indicating the endogenous protein (lower band) and the FLAG-tagged exogenous protein (upper band). Overexpression of ILK was shown to increase TWIST by 1.9-fold at the protein level. (**b**) SKBR3^{vector} and SKBR3^{ILKWT} cells were grown on coverslips, fixed with 4% paraformaldehyde (PFA) SKBR3^{ILKWT} and then stained for TWIST (red). Nuclei were counterstained with Hoechst (blue). Immunofluorescent analysis showed that SKBR3^{ILKWT} cells have a substantial increase in TWIST staining. (**c**) Protein lysates were collected from SKBR3^{vector} and SKBR3^{ILKWT} cells, cytoplasmic fractions were separated from nuclear fractions and run out on SDS–PAGE gels. The resulting western analysis showed that P-STAT-3^{se705} increased by twofold in the nuclear fraction of cells transfected with ILK. Interestingly ILK overexpressing cells exhibit a 225% increase in total STAT when considering cytoplasmic and nuclear fractions together in cells transfected with ILK when compared with vector-transfected cells.

2006; Fujii *et al.*, 2008; Kashihara *et al.*, 2009). Furthermore, it has been shown that knockdown of YB-1 with siRNA reduces Her2/*neu* expression (Lo *et al.*, 2007). RNA interference strategies targeting ILK have been shown to interfere with nuclear translocation of YB-1 in human ovarian cancer cells (Basaki *et al.*, 2007), and it was postulated that this effect occurred through decreased phosphorylation of AKT. Thus, inhibition of ILK and associated suppression of phosphorylation of AKT could act to maintain levels of YB-1 in the cytoplasm. In the cytoplasm, YB-1 can act as a translation factor binding to mRNA (Ozer *et al.*, 1990; Chernov *et al.*, 2008a, b, 2009). Messenger RNA is normally bound to proteins, forming polysomes and mRNPs. Messenger RNA released from disassembled polysomes and mRNPs are sorted and remodeled in stress granules (SGs), from which selected transcripts are delivered to processing bodies for degradation. SGs are cytoplasmic aggregates of protein and RNA approximately 100–200 nm in diameter and they are thought to be sites of stalled translation (for example, pre-initiation complexes; Yamasaki and Anderson, 2008; Balagopal and Parker, 2009; Anderson and Kedersha, 2009a, b). Furthermore, Kedersha and Anderson (2007) established that YB-1 is a useful marker of SGs and processing bodies and YB-1 modulates the formation of SGs and translation of mRNA.

In this study, using immunofluorescent localization of YB-1 in SKBR3 cells, we demonstrated decreases in YB-1 nuclear staining and cytosolic sequestration of YB-1 into intracellular puncta that could be SGs following treatment with QLT0267 (see Figure 4c). This effect was also observed in the other breast cancer cell lines that were studied here. The formation of SGs may have a role in the translational regulation of both Her2/*neu* and YB-1. As suggested above, decreases in phosphorylation of AKT (serine 473) caused by ILK inhibition or silencing could lead to accumulation of YB-1 in the cytoplasm where it may form SGs and processing bodies, leading to the degradation of Her2/*neu* and

YB-1 transcript. The results presented in Figure 2 demonstrated decreases in P-AKT^{ser473} following treatment with QLT0267 or ILK silencing by siRNA. Although this effect was transient, it remains a possible mechanism through which ILK may regulate the localization of YB-1 and thus impact Her2/*neu* expression. It is important to note that previous reports suggest that the LTR-RSV promoter used to drive c-*erbB2* gene expression in the MCF7 and LCC6 cells has a binding site for YB-1 (Ozer *et al.*, 1990), which could also support a transcriptional regulatory mechanism of Her2/*neu* via ILK and YB-1.

YB-1 is also associated with increased expression of EGFR. Thus, as a follow-up to the studies evaluating Her2/*neu* signaling, EGFR expression was analyzed after treatment with QLT0267. Our data show that total EGFR expression in SKBR3 cells is significantly decreased by approximately 87% when using QLT0267 when compared with vehicle control (that is, PTE (polyethylene glycol, Tween 80, 95% ethanol and citric acid) see Supplementary Figure 4). These studies have interesting implications, particularly with the use of ILK inhibition or silencing in combination with EGFR and Her2/*neu* inhibitors. Furthermore, cell viability experiments were initiated to evaluate combinations of QLT0267 with Lapatinib, a dual kinase small molecule inhibitor targeting Her2/neu and EGFR. Our preliminary results indicate that at low effect levels the interactions between QLT0267 and Lapatinib are synergistic, but at the desirable high effect levels the QLT0267/Lapatinib interactions are strongly antagonistic, perhaps owing to the loss of EGFR and Her2/*neu* expression with higher doses of QLT0267.

To elucidate a mechanism of transcriptional regulation of YB-1 via ILK, the studies described here also evaluated the transcription factor TWIST. TWIST is known to bind to E-box regions within the YB-1 promoter and thus regulate its expression (Shiota *et al.*, 2008a, b, 2009). TWIST is considered oncogenic and is overexpressed in breast cancer (Watanabe *et al.*, 2004; Martin *et al.*, 2005). Entirely consistent with a role for TWIST in ILK-mediated regulation of Her2/*neu* expression, decreased ILK expression or activity led to a near-complete inhibition of TWIST expression (Figure 5a), suggesting that ILK may be able to regulate TWIST expression. Moreover, silencing of TWIST was associated with decreased expression of both YB-1 and Her2/*neu* (see Figure 5b). Finally, overexpression of ILK in SKBR3 cells resulted in increased TWIST expression (see Figures 6a and b), suggesting for the first time that TWIST is a downstream target of ILK. Preliminary studies are already underway to determine whether transfection of cells with a YB-1 construct is able to rescue Her2/*neu* expression and in the same vein it would be interesting to determine whether forced overexpression of TWIST could potentially recover YB-1 and Her2/*neu* expression after ILK inhibition.

Figure 7 details the possible pathways through which ILK may modulate the expression and activity of TWIST and therefore YB-1 and Her2/*neu*. TWIST expression is known to be regulated by two transcription factors that can be directly linked to the pathways influenced, in part, by ILK. These include STAT-3 (Ling and Arlinghaus, 2005; Lo *et al.*, 2007; Cheng *et al.*, 2008b) and hypoxia-inducible factor-α (Gort *et al.*, 2008; Peinado and Cano, 2008; Yang and Wu, 2008; Yang *et al.*, 2008). In this paper we examine the role of ILK in the activation of STAT-3. We show that ILK overexpression is associated with increased levels of phosphorylated and thus activated STAT-3 (Figure 6c). Activation of STAT-3 allows for its nuclear translocation and thus induction of gene transcription. Where ILK activity or expression is attenuated, this pathway is shut down. Inactive STAT-3 is no longer able to promote transcription of TWIST, which thereafter is unable to initiate transcription of YB-1. It is interesting to note that phenotypic changes seen with TWIST overexpression mimic those seen with ILK overexpression and include increased epithelial–mesenchymal transition (Karreth and Tuveson, 2004; Ansieau *et al.*, 2008; Cates *et al.*, 2009), increased VEGF secretion (Mironchik *et al.*, 2005; Niu *et al.*, 2007), increased propensity for invasion and migration (Karreth and Tuveson, 2004; Elias *et al.*, 2005; Luo *et al.*, 2008; Cheng *et al.*, 2008a; Matsuo *et al.*, 2009; Valdes-Mora *et al.*, 2009), evasion of apoptosis (Maestro *et al.*, 1999; Dupont *et al.*, 2001; Zhang *et al.*, 2007), drug resistance (Kajiyama *et al.*, 2007; Pham *et al.*, 2007; Zhuo *et al.*, 2008) and deregulated growth (Maestro *et al.*, 1999; Kwok *et al.*, 2005; Puisieux *et al.*, 2006; Hu *et al.*, 2008; Shiota *et al.*, 2008a; Hasselblatt *et al.*, 2009). TWIST has been labeled as the master regulator of epithelial–mesenchymal transition, and thus the previously unrecognized role of ILK in regulating TWIST expression is very relevant in the context of managing cancer development and progression. Studies are now underway to further explore the relationship between ILK and TWIST *in vivo*.

Conclusion
This study shows for the first time that ILK can regulate the expression of Her2/*neu* through a pathway that involves TWIST and YB-1. The broader implication of this study is support for the use of ILK inhibitors in the treatment of aggressive Her2/*neu*-positive tumors.

Materials and methods

Chemicals and reagents
QLT0267 (267) was a generous gift from QLT Inc. (Vancouver, BC, Canada) and was diluted in PTE. QLT0267 is a second-generation ILK inhibitor derived from KP-392. Among 150 kinases tested, QLT0267 is highly specific, showing 1000-fold selectivity over kinases including CK2, CSK, DNA-PK, PIM-1, PKB/Akt and PKC, and 100-fold selectivity over other kinases such as Erk-1, GSK-3β, LCK, PKA, p70S6K and RSK1 (Troussard *et al.*, 2006; Younes *et al.*, 2007). It has also been established that the effects of QLT0267 are similar to those of dominant-negative ILK mutants and to the effects seen using ILK-targeted siRNA sequences. QLT0267 was evaluated as being more potent than KP-392 and was found to inhibit ILK kinase activity in cell-free systems at 26 nmol/l (QLT Inc., unpublished data). These
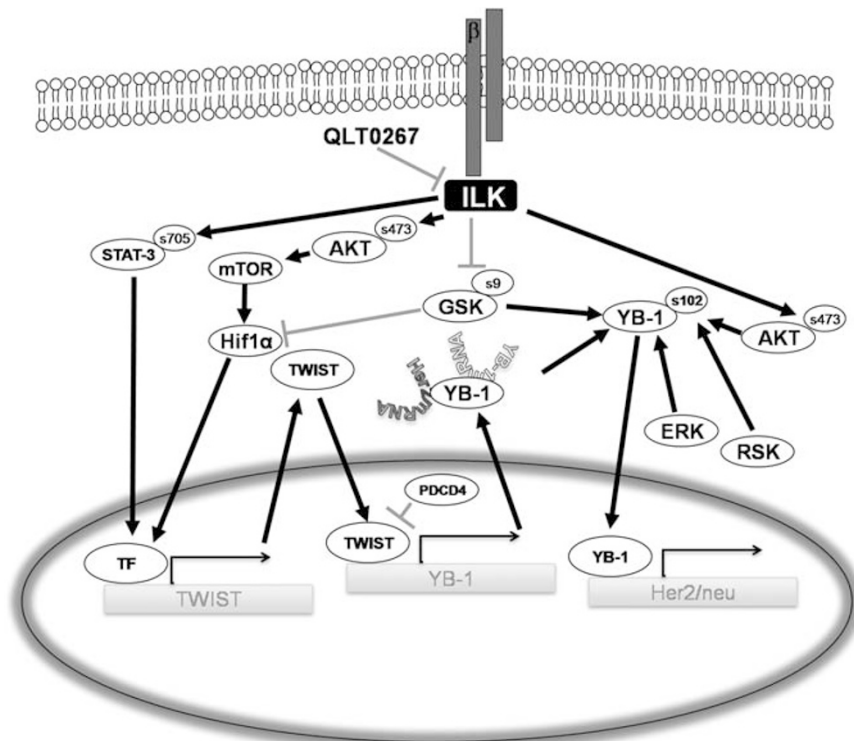
Figure 7 Proposed working model of the ILK-centered regulation of Her2/*neu* through multiple mechanisms involving YB-1. ILK is able to phosphorylate many downstream effectors that have the potential to regulate YB-1 transcription, translation and subcellular localization. Known kinases that phosphorylate YB-1 inducing its nuclear translocation include GSK-3 (Coles *et al.*, 2005), ERK (Coles *et al.*, 2005), RSK (Stratford *et al.*, 2008) and AKT (Sutherland *et al.*, 2005; Oda *et al.*, 2007; To *et al.*, 2007; Bader and Vogt, 2008). ILK activates AKT through phosphorylation of serine 473 (Delcommenne *et al.*, 1998; Persad *et al.*, 2000; McDonald *et al.*, 2008a, b). ILK inhibits GSK-3β through phosphorylation on serine 9 (Delcommenne *et al.*, 1998; Troussard *et al.*, 1999). When active, GSK-3 ubiquinates hypoxia-inducible factor-1α (Hif1α), leading to its degradation (Mottet *et al.*, 2003; Flugel *et al.*, 2007). However, when Hif1α is activated, which can occur through AKT/mTOR (Pore *et al.*, 2006), it translocates to the nucleus and can bind to the HRE segment on TWIST promoter, leading to an increase in TWIST protein (Gort *et al.*, 2008; Peinado and Cano, 2008; Yang and Wu, 2008; Yang *et al.*, 2008). Thus, ILK can regulate the expression of TWIST by phosphorylating AKT, leading to activation of Hif1α. Alternatively, ILK can also phosphorylate STAT-3 on serine 705 (Yau *et al.*, 2005; Fuchs *et al.*, 2008). Activated STAT-3 translocates to the nucleus and induces the expression of TWIST (Ling and Arlinghaus, 2005; Lo *et al.*, 2007; Cheng *et al.*, 2008b). TWIST binds to E-box regions in the promoter sequence of YB-1 initiating its transcription (Shiota *et al.*, 2008a, b, 2009). YB-1 nuclear localization is associated with increased expression of Her2/*neu* (Wu *et al.*, 2006; Fujii *et al.*, 2008; Kashihara *et al.*, 2009). When ILK is inhibited, several of these pathways could potentially lead to decreased transcription of YB-1 and cytosolic sequestration in stress granules. When YB-1 is depleted in the nucleus, transcription of Her2/*neu* is decreased (Wu *et al.*, 2006; Fujii *et al.*, 2008; Kashihara *et al.*, 2009). In the cytoplasm, YB-1 may bind to YB-1 and Her2/*neu* mRNA, inhibiting their translation (Skabkina *et al.*, 2003, 2004, 2005).

studies were preformed using highly purified recombinant ILK, and the lower concentrations of QLT0267 required to inhibit ILK in these cell-free systems likely reflects the fact that QLT0267 is not very membrane permeable. Moreover, the *in vitro* activity of QLT0267 is dependent on the cell line being used, its metabolism and growth, among other factors. This is consistent with *in vitro* studies using other small molecule inhibitors. Previous work done by our lab and others has shown that optimal concentrations of QLT0267, which inhibits activation of downstream effectors of ILK, occur in cells between 1 and 50 μM (Supplementary Figure 1).

Despite the specificity of the QLT0267 small molecule inhibitor, we were concerned that the concentrations used may elicit off target effects, and for this reason silencing of ILK expression via siRNA was used to confirm results obtained with QLT0267. Negative control siRNA (low guanine–cytosine content; Neg), siRNA sequences against human TWIST1 mRNA (Genbank accession no. NC:000007) and ILK mRNA (Genbank accession no. GI:3150001) were generated by Invitrogen (Burlington, ON, Canada).

The pIRES-hrGFP (Stratagene, La Jolla, CA, USA) vectors containing a FLAG-tagged full-length human normal ILK (*ILKWT*) gene were a generous gift from Dr Shoukat Dedhar. All other chemicals, unless specified, were purchased from Sigma Chemical Company (Oakville, ON, Canada).

Cells and cell culture
All cell lines were tested to ensure that they were mycoplasma free. Cells used for studies were derived from original stocks that had been expanded and frozen. They were maintained in culture for no more than 20 passages and at that time were replaced with frozen stock. MDA-MB-435/LCC6 (Leonessa *et al.*, 1996) breast cancer cells were a gift from Dr Robert Clarke (Georgetown University, Washington, DC, USA) and were derived from the parental cell line MDA-MB-435. The origin of this cell line is controversial (Chambers, 2009) but we believe it is justifiable to use these cells as a model breast cancer cell line (Dragowska *et al.*, 2004). LCC6 cells were transfected via electroporation with the mammalian expression vector

pREP9 (Invitrogen, Grand Island, NY, USA) containing the 4.3 kb full-length human normal c-*erbB2* complementary DNA to yield LCC6[Her2] as previously described (Dragowska *et al.*, 2004; Warburton *et al.*, 2004). JIMT-1 cells were a gift from Dr Jorma Isola (Tampere University and Tampere University Hospital, Tampere, Finland). MCF7, KPL-4 and SKBR3 cells were purchased from American Type Culture Collection (Manassas, VA, USA). MCF7[Her2] cells were a kind gift from Dr Moulay Alaoui-Jamali (McGill University, Montreal, Quebec, Canada). LCC6, JIMT-1, BT474, KPL-4, MCF7[Her2] and MCF7 cells were maintained in Dulbecco's modied Eagle's medium/high glucose supplemented with L-glutamine (Stem Cell Technologies, Vancouver, BC, Canada) and 10% fetal bovine serum (Hyclone, Logan, UT, USA). SKBR3 cells were maintained in McCoy's 5a medium (Stem Cell Technologies) supplemented with L-glutamine and 10% fetal bovine serum. MCF7 cells were nucleofected (Amaxa Biosystems, Lonza Cologne, Basel, Switzerland) with the pIRES-hrGFP empty vector or the vector containing *ILKW*T gene and selected with G418 and sorted for green fluorescent protein (GFP) using fluorescence-activated cell sorting. All cells were maintained at 37 °C and 5% CO_2 in a humidified atmosphere.

Nucleofection of siRNA or plasmid DNA
SKBR3, LCC6[Her2] and JIMT-1 cells were transiently nucleofected with ILK siRNA as previously described (Verreault and Bally, 2009). MCF7 and SKBR3 cells were stably nucleofected with a plasmid encoding ILKWT. The nucleofection protocols for siRNA and plasmid DNA were similar. Briefly, the Nucleofector technology (Amaxa Biosystems) was used according to the manufacturer's protocol. Optimal conditions were first determined using a GFP plasmid or Cy5 siRNA and analysis of cell labeling by FLOW cytometry. Once the protocols were defined, 1×10^6 cells were suspended in nucleofection buffer containing 1–4 μg of either siRNA or plasmid DNA and placed in the nucleofector for electroporation. Programs E09 (buffer C), D010 (buffer R), T020 (buffer R) and P020 (buffer R) were used in SKBR3, LCC6[Her2] JIMT-1 and MCF7 cells, respectively. Cells were re-suspended in Dulbecco's modied Eagle's medium and allowed to recover at 37 °C and 5% CO_2 in a humidified atmosphere. For plasmid nucleofection, cells were selected for using G418 (Invitrogen, Canada) and sorted based on the expression of GFP. Cells were analyzed for GFP expression using FLOW cytometry before each use. Cells that were >90% positive for GFP were processed for subsequent analyses.

SDS–PAGE and western blot
Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by western blotting analysis was used to semiquantitatively determine ILK, AKT, pAKT TWIST, YB-1 and Her2/*neu* protein levels. Briefly, whole-cell lysates were harvested by incubation in lysis buffer (50 mM Tris/HCl pH 8.5, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP-40 and 0.5% sodium deoxycholate) and pelleted by centrifugation at 14 000 r.p.m. for 10 min at 4 °C. For subcellular localization studies, cells were incubated in lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCL, 1 mM EDTA and 0.1% NP40) and then pelleted. The supernatant was collected as the cytoplasmic fraction, and the pellet was re-suspended in extraction buffer (0.42 mM NaCl, 20 mM HEPES, 1.5 mM $MgCl_2$ and 20% glycerol). Cells were pelleted and the supernatant was collected as the nuclear fraction. Samples were separated on 10% SDS–PAGE gels. Protein was transferred to Nitrocellulose membrane (Millipore,

Bedford, MA, USA) and blocked in Odyssey blocking buffer (Licor Biosciences, Lincoln, NE, USA). The blots were labeled with mouse polyclonal anti-ILK (Transduction Laboratories, BD Biosciences, Franklin Lakes, NJ, USA), rabbit polyclonal anti-TWIST, anti-AKT, anti-pAKT[ser473] anti-pSTAT[ser705] anti-Her2/*neu*, mouse monoclonal anti-heat shock protein 90 (Cell Signaling Technology, Beverly, MA, USA), rabbit polyclonal anti-YB-1 (Abcam, Cambridge, MA, USA) or rabbit polyclonal anti-CREB (Millipore (Upstate), Etobicoke, ON, USA) antibodies. Primary antibody binding was detected by further incubations with anti-rabbit IRDYE (green; Rockland, Gilbertsville, PA, USA) or anti-mouse Alexa 680 (red) (Invitrogen, Molecular Probes, Burlington, ON, Canada) and signal was detected and quantified using the Odyssey Infrared Detection System and associated software (Odyssey v1.2; Licor). Protein loading was determined by re-probing membranes for β-actin (Sigma-Aldrich, Oakville, ON, Canada). The absorbance of specific protein bands in a square region of interest surrounding each band, after background subtraction, was normalized to actin bands measured in the same way. Studies were conducted at least three times. Where indicated, absorbance data were expressed as mean absorbance values ± s.d. and parametric analysis was done using an unpaired Student's *t*-test.

Immunofluorescent imaging
Cells grown on coverslips were fixed using a 2.5% paraformaldehyde solution in phosphate-buffered saline (PBS), permeabilized with Triton X-100 and blocked in a 2.5% bovine serum albumin solution in PBS for 1 h at room temperature before staining for YB-1 using a polyclonal rabbit primary antibody (1:25) or TWIST, a polyclonal rabbit primary antibody (1:50). All antibodies were diluted in bovine serum albumin/PBS. Coverslips were washed three times for 5 min using PBS. Primary antibody binding was detected by further incubations with anti-rabbit Alexa546 or Alexa488. To ensure that there was no nonspecific antibody binding, a secondary antibody control coverslip was used for each experiment where coverslips were stained with either Alexa546 or Alexa488 alone (data not shown). Hoechst (Molecular Probes, Eugene, OR, USA; 1:1000) was used to identify nuclei. The coverslips were then mounted to a microscope slide using a 9:1 solution of glycerol and 1 × PBS. Cells were viewed using a Leica fluorescent microscope (Wetzlar, Germany) with a × 100 oil immersion lens under the Z568RDCf filter set (Chroma, Rockingham, VT, USA) to visualize Alexa 546 and ultraviolet lamp to visualize Hoechst. Images were captured using DC100 digital camera and Open Lab software (Improvision, Lexington, MA, USA).

Dual luciferase reporter assay
SKBR3 cells were plated in six-well plates (4×10^5 cells/well) and transfected with a luciferase construct (pGL3 basic vector; Promega, Madison, WI, USA) containing the core promoter and the partial first exon of the YB-1 gene (courtesy of Dr Kimitoshi Kohno, Department of Molecular Biology, University of Occupational and Environmental Health, Kitakyushi, Japan). Cells were transfected with a total of 1.0 μg DNA using FuGene (Roche, Toronto, ON, Canada). To assess transfection efficiency, cells were co-transfected with a *Renilla*-expressing plasmid (pRL-thymidine kinase, 10:1 luciferase:*Renilla*; Promega). After 24 h, cells were treated with vehicle (PTE) or QLT0267 (42 μM) for 24 h before harvesting in 1 × passive lysis buffer (Promega). Luciferase activity was measured using the Lumat LB 9507 Luminometer (Berthold

Technologies, Oak Ridge, TN, USA) according to the manufacturer's instructions, and results were normalized to the corresponding *Renilla* readings from the same sample. The studies were done at least three times and luminescence data were expressed as mean values ± s.d. and parametric analysis was done using an unpaired Student's *t*-test.

RNA extraction and PCR
The extraction of total RNA from a minimum of 1×10^6 cells was completed using the RNeasy mini kit (Qiagen, Hilden, Germany). RNAs were reverse transcribed using the Invitrogen Superscript III kit (Invitrogen, Canada) according to the manufacturer's instructions. The RNA concentration of each sample was measured using the Nanodrop ND1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and sample purity was determined by assessing the A_{260}/A_{280} ratio, which always measured between 2.0 and 2.1. For PCR, 1–3 µg of complementary DNA was added to PCR master mix containing $10 \times$ PCR buffer, 3 mM $MgCl_2$, 1 mM dNTP, Taq polymerase and 0.5 µM each of the appropriate forward and reverse primers (Invitrogen, Canada) where GAPDH mRNA was used as an internal standard. Primer sequences used were as follows:

Her2/*neu* forward primer 5′-TCCTGTGTGGACCTGGA TGAC-3′
Her2/*neu* reverse primer 5′-CCAAAGACCACCCCCA AGA-3′
YB-1 forward primer 5′-AAGTGATGGAGGGTGCTG AC-3′
YB-1 reverse primer 5′-TTCTTCATTGCCGTCCTCTC-3′
GAPDH forward primer 5′-GAAGGTGAAGGTCGGA GT-3′
GAPDH reverse primer 5′-GAAGATGGTGARGGGAT TTC-3′

Complementary DNA was amplified using the DNA engine Peltier thermal cycler (Bio-Rad, Mississauga, ON, Canada).

PCR products were run out on a 1.5% agarose gel containing 0.004% ethidium bromide, and detected using the Eagle Eye II Cabinet detection system (Stratagene).

Quantitative real-time PCR
Quantitative SYBR green PCR assays for YB-1 and Her2/*neu* was performed in a ABI Prism 7700 Sequence detection system (Applied Biosystems, Streetsville ON, Canada) using the SYBR Green Kit supplied by Applied Biosystems. PCR amplification were carried out in a 20 µl volume under the following conditions: an enzyme activation step at 95 °C for 2 min, followed by 45 cycles consisting of 30 s of denaturation at 95 °C, 20 s of annealing at 60 °C and 20 s of elongation at 72 °C. The specificity of the amplified products was verified by melting curve analysis and agarose gel electrophoresis. Ct values were converted to fold change.

Conflict of interest

The authors declare no conflict of interest.

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