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LMO4 is an essential mediator of ErbB2/HER2/Neu-induced breast cancer cell cycle progression

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Abstract

ErbB2/HER2/Neu-overexpressing breast cancers are characterized by poor survival due to high proliferation and metastasis rates and identifying downstream targets of ErbB2 should facilitate developing novel therapies for this disease. Gene expression profiling revealed the transcriptional regulator LIM-only protein 4 [LMO4] is upregulated during ErbB2-induced mouse mammary gland tumorigenesis. While LMO4 is frequently overexpressed in breast cancer and LMO4-overexpressing mice develop mammary epithelial tumors, the mechanisms involved are unknown. Herein, we report that LMO4 is a downstream target of ErbB2 and PI3K in ErbB2-dependent breast cancer cells. Furthermore, LMO4 silencing reduces proliferation of these cells, inducing a G2/M arrest that was associated with decreased cullin-3, an E3-ubiquitin ligase component important for mitosis. Loss of LMO4 subsequently results in reduced Cyclin D1 and Cyclin E. Further supporting a role for LMO4 in modulating proliferation by regulating cullin-3 expression, we found that LMO4 expression oscillates throughout the cell cycle with maximum expression occurring during G2/M and these changes precede oscillations in cullin-3 levels. LMO4 levels are also highest in high grade/less differentiated breast cancers, which are characteristically highly proliferative. We conclude that LMO4 is a novel cell cycle regulator with a key role in mediating ErbB2-induced proliferation, a hallmark of ErbB2-positive disease.

Keywords

Breast Cancer; cullin-3; ErbB2; HER2; LMO4

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Introduction

The *ErbB2/HER2/Neu* gene is amplified in 15–30% of invasive breast cancers and this correlates with high grade tumors that are highly proliferative and convey poor outcome (Slamon et al., 1989). Currently, the most effective therapy for this subtype of breast cancer is trastuzumab, a humanized monoclonal antibody that binds the extracellular domain of ErbB2 and inhibits receptor signaling. Although this therapy has proven successful for ∼50% of patients with ErbB2-positive disease, many patients are resistant or develop resistance to this drug (Romond et al., 2005; Piccart-Gebhart et al., 2005). Furthermore, among those patients treated with trastuzumab, a significant proportion must discontinue treatment due to cardiotoxic side-effects (Seidman et al., 2002). Besides the clinical limitations of trastuzumab, tumor studies in mice have revealed that recurrence occurs after complete suppression of ErbB2 even though it initiated the primary tumors (Moody et al., 2005). This suggests that activation of additional autonomous oncogenic signals contributes to tumor cell survival and progression. Hence, identification of novel downstream targets of ErbB2 may be necessary to develop new treatment options.

ErbB2 is a member of the EGFR tyrosine kinase family, which also includes ErbB3 and ErbB4 (Yamamoto et al., 1986). Although ErbB2 does not bind any known ligand, growth factor binding to other family members induces ErbB2 activity because it is the preferred heterodimerization partner (Graus-Porta et al., 1997). Signaling via ErbB2 activates several major signaling pathways including MAPK (Ben Levy et al., 1994), PI3K/AKT (Peles et al., 1992), and PLCγ (Peles et al., 1991). These pathways induce proliferation, in part by upregulating the cell cycle protein, Cyclin D1 (Lenferink et al., 2001), which is essential for ErbB2-induced transformation (Yu et al., 2001; Lee et al., 2000). While several breakthroughs have been made in identifying early signaling events initiated by ErbB2, many of the downstream effectors of ErbB2-induced tumorigenesis remain unknown. Identification of these intermediates should provide significant insight into the molecular mechanisms employed by ErbB2 to induce breast cancer.

Correlative studies have indicated that the LIM-only protein 4 [LMO4] is overexpressed in the majority of breast cancers that also have ErbB2 amplification (Sum et al., 2005b). LMO4 belongs to the LIM-only family, which is characterized by two tandem, non-DNA binding LIM domains that confer the potential to interact with multiple proteins simultaneously (Rabbitts, 1998). Because LMO proteins bind to transcription factors and cofactors, they have been implicated in the control of gene expression by forming multimeric transcriptional complexes (Sum et al., 2002).

Conditional loss of *LMO4* in the mammary gland results in reduced lobuloalveolar development with a concomitant decrease in cellular proliferation (Sum et al., 2005c; Wang et al., 2007). These findings suggest that LMO4 is necessary for expansion of the epithelial compartment of the mammary gland, possibly due to its regulation of proliferation. Supporting this notion, targeted overexpression of LMO4 in mouse mammary glands induces hyperplasia and tumors (Sum et al., 2005b). *In vitro*, overexpression of LMO4 or its binding partner Ldb1, inhibited expression of differentiation markers in mammary epithelial

cells (Visvader et al., 2001), while LMO4 silencing is associated with decreased growth, migration and invasion of breast cancer cells (Sum et al., 2005b). Although these data implicate LMO4 in the pathogenesis of breast cancer through its modulation of proliferation, the mechanisms by which LMO4 regulates proliferation and the processes that control LMO4 expression and activity remain unknown.

In human breast cancers, LMO4 is overexpressed in 56% of all breast tumors (Visvader et al., 2001), and 65% of those that are positive for ErbB2 (Sum et al., 2005b). *LMO4* mRNA is also elevated in several other cancers including prostate cancers (Mousses et al., 2002) and small cell lung carcinomas that fail chemotherapy (Taniwaki et al., 2006). Most importantly, multivariate analysis of human breast cancers suggests that LMO4 overexpression may account for much of the aggressiveness associated with ErbB2-overexpression (Sum et al., 2005b). However, a functional link between LMO4 and ErbB2 has yet to be identified.

Herein, we report that ErbB2 and its downstream signaling protein, PI3K, regulate LMO4 expression in breast cancer cells. Furthermore, LMO4 is necessary for ErbB2-mediated proliferation and cell cycle progression. LMO4 expression fluctuates throughout the cell cycle and is required for sustained expression of key cell cycle regulators: the G1 cyclin, Cyclin D1, and the G2/M ubiquitin E3-ligase component, cullin-3. We conclude LMO4 is an essential downstream effector of ErbB2-induced cell cycle progression and likely plays an important role in modulating the aggressiveness of various types of breast cancer.

Results and Discussion

LMO4 mRNA and protein are increased in ErbB2-induced tumors

We previously described the use of gene expression profiling to identify an ErbB2-induced "tumor signature" using the mouse model of breast cancer, MMTV-c-*Neu*, which overexpress ErbB2 in the mammary glands and develop tumors with mean latency of 10 months (Guy et al., 1992; Landis et al., 2005). Comparison of tumors and hyperplastic mammary glands to age-matched wild-type mammary glands revealed several differentially regulated genes. Among these, *LMO4* mRNA expression was elevated in MMTV-c-*Neu* hyperplastic tissue relative to wild-type glands and was further increased in MMTV-c-*Neu* tumors (Landis et al., 2005), indicating that *LMO4* gene expression gradually increases during the progression from pre-neoplastic glands to overt tumors (Figure 1a). Tumors showed ∼5 fold increase in *LMO4* mRNA levels compared to wild-type control glands. The presence of *LMO4* mRNA was confirmed in an additional set of tumors by *in situ* hybridization. Expression of *LMO4* mRNA occurred throughout the entire tumor suggesting most ErbB2-overexpressing tumor cells also overexpress LMO4 (Figure 1b). Furthermore, elevated LMO4 protein expression in tumors compared to wild-type glands was confirmed in additional samples (Figure 1c). Comparison of *LMO4* expression across several murine mammary gland tumor models (Kuraguchi et al., 2009; Desai et al., 2002) revealed that *LMO4* mRNA is preferentially upregulated in tumors that overexpress ErbB2/Neu, polyoma middle T antigen and HA-ras (Table 1). Since pathways initiated by these oncogenes show high degree of convergence (Dankort et al., 2000), these data indicate that elevated LMO4

expression in mouse mammary tumors is selectively associated with activation of ErbB2 and its downstream signaling pathways.

ErbB2 and PI3K activity are necessary for LMO4 expression

The coordinated increase in LMO4 expression that occurs with ErbB2 activation suggested that ErbB2 signaling may induce LMO4 expression. To characterize the mechanisms involved in upregulation of LMO4 in ErbB2-overexpressing tumors, we used various downstream pathway inhibitors in BT-474 breast cancer cells. This cell line overexpresses endogenous ErbB2 (Kraus et al., 1987), serving as an *in vitro* model to study ErbB2-induced carcinogenesis. To determine which signaling pathways may impact LMO4 expression, these cells were treated with inhibitors of EGFR (ZD1836), ErbB2 (AG825), PI3K (LY294002), MEK (PD98059), or JNK (JNK Inh II). Inhibition of EGFR, ErbB2 or PI3K led to a 28%, 86% or 75% decrease in LMO4 protein expression, respectively (Figure 2a/b, Supplemental Figure S1b). In contrast, inhibition of MEK or JNK had no effect on LMO4 protein levels. All inhibitor treatments blocked their respective pathways as indicated by changes in the phosphorylation status of various targets (Supplemental Figure S1a). To determine if the changes in LMO4 protein were the result of changes in gene expression, we analyzed *LMO4* mRNA levels by qRT-PCR. Similar to the changes in protein, *LMO4* mRNA expression was decreased by 80% upon inhibition of ErbB2 activity (Figure 2c), indicating that the changes in LMO4 protein expression that occur in response to altering ErbB2 activity are largely the result of changes in mRNA levels. Taken together, these data suggest that *LMO4* gene expression is dependent on activation of ErbB2-containing hetero- or homodimers and their subsequent activation of PI3K. Surprisingly, LMO4 expression is not dependent upon AKT because modulation of AKT activity with a small molecule inhibitor, or transfection with dominant negative or constitutively active forms of AKT had no effect on LMO4 protein expression (Figure 2d). The dependency on PI3K, but not AKT, suggests that other targets of PIP_3 such as aPKC, PLC or integrin-linked kinase [ILK] may be involved in modulating LMO4 expression.

The *LMO4* gene is not a primary target for somatic mutations (Sutherland et al., 2003) and is not rearranged or amplified in breast cancer cells (Wittlin et al., 2003). These findings, coupled with the requirement for sustained ErbB2 signaling to maintain *LMO4* mRNA levels, suggested that ErbB2 may regulate *LMO4* transcription. The *LMO4* gene contains two promoters and 6 exons (Figure 2e) that generate a single protein (Wittlin et al., 2003). The two promoters generate two different mRNAs: one -containing exons 1a-5 (minus 1b) and another lacking exon 1a but containing exons 1b-5 (Wittlin et al., 2003). To assess transcriptional control of the *LMO4* gene by ErbB2 signaling, transient transfection analyses were performed with 5' deletion constructs of promoter 1 (P1) and promoter 2 (P2) linked to luciferase. Activities of the two promoters were assessed in BT-474 cells in the presence or absence of inhibitors of the ErbB2 signaling cascade (Figure 2e). Only the proximal promoter (P2) was responsive to ErbB2 inhibition, exhibiting a 50% decrease in activity with AG825, a selective ErbB2 inhibitor. In contrast, the selective PI3K inhibitor (LY294002) suppressed both fragments obtained from the distal (P1) promoter, P1–1300/+34 and P1–100/+34, by 61% and 53%, respectively, as well as the proximal (P2) promoter by 76%. While only the proximal promoter of the *LMO4* gene contains an ErbB2

responsive domain, these data indicate that multiple PI3K responsive elements reside within promoter regulatory regions with at least one element occurring in each of the distal and proximal promoters. The differential effect of the ErbB2 and PI3K inhibitors on the two promoter regions suggests that they may be under distinct control, with signaling factors other than ErbB2 that are upstream of PI3K regulating the *LMO4* distal promoter. Indeed, the ErbB2 inhibitor only decreases a fraction of the active PI3K in the cell at any given time (Supplemental Figure S1a) and other active receptors in the cell, such as the insulin receptor (Sanchez-Margalet et al., 1994), may still signal through PI3K.

To further delineate the mechanisms by which ErbB2 regulates LMO4 expression, ErbB2 was overexpressed in MCF-7 breast cancer cells, which have low ErbB2 levels. Overexpression of ErbB2 results in 2.7 fold induction of LMO4 protein (Supplemental Figure S2a/b). The increase in LMO4 protein is associated with 1.7 fold induction of the *LMO4* proximal promoter as well as a slight increase in activity from the P1–100/+34 distal promoter construct (Supplemental Figure S2c). Comparison of these ErbB2-induced changes revealed that much of the increase in LMO4 protein expression can be explained by an increase in *LMO4* promoter activity. However, the lack of complete concordance between promoter activity and protein levels indicates that ErbB2 may utilize additional mechanisms of LMO4 regulation such as changes in mRNA stability (Chen et al., 2007) or use of additional promoter regulatory regions.

Although LMO4 overexpression is frequently observed in primary breast cancers, the signaling cascades that control its expression have remained elusive. Our data suggest that LMO4 is a novel downstream target of the ErbB2 signaling cascade that proceeds through the PI3K signaling arm. The ability of ErbB2 to induce LMO4 is supported by correlative clinical data in which overexpression of LMO4 is seen in 65% of ErbB2 positive breast cancers (Sum et al., 2005b) and *in vitro* data in which treatment of MCF-7 breast cancer cells with heregulin, an ErbB agonist, elevates *LMO4* mRNA (Wang et al., 2004). Furthermore, the requirement for both LMO4 (Wang et al., 2004; Sum et al., 2005c) and ErbB2 (Andrechek et al., 2005) in normal mammary gland development and the ability of these two factors to induce mammary cancer when overexpressed points to the possibility that LMO4 may be an intermediary of ErbB2.

LMO4 regulates proliferation of ErbB2-dependent breast cancer cells

LMO4 promotes the growth of multiple breast cancer cell lines (Sum et al., 2005b). Thus, we postulated that LMO4 may also be required for ErbB2-induced proliferation. To silence *LMO4* gene expression, transient transfections were performed with either non-silencing siRNA (NS) or siRNA directed to LMO4 (siLMO4#3) in BT-474 and SKBR3 cells, both of which rely on sustained ErbB2 expression for proliferation (Yakes et al., 2002). LMO4-directed siRNA reduced LMO4 protein levels by ∼90 % in BT-474 (Figure 3a) and SKBR3 cells (Figure 3c). BrdU incorporation was then used to identify proliferating cells. Knock-down of LMO4 resulted in a ∼40% decrease in the percent of BrdU-positive cells in both cell lines (Figure 3 b/d), suggesting that LMO4 is necessary to sustain proliferation in ErbB2-dependent breast cancer cells. The requirement for LMO4 for proliferation is consistent with its expression pattern in the mouse mammary gland where it is highly

expressed in cap cells and in alveolar cells during development, two sites with elevated proliferation rates (Sum et al., 2005a). LMO4 is expressed in highly proliferating epithelial cells of other tissues as well (Sum et al., 2005a; Sugihara et al., 1998). Our data extends these results by showing that LMO4 expression is not only regulated by ErbB2 activity, but that it is also required for proliferation of breast cancer cells that are dependent on ErbB2 signaling.

LMO4 is required for maintenance of Cyclin D1 mRNA and protein expression in cells with constitutive ErbB2 signaling as well as for heregulin-induced upregulation of Cyclin D1

To identify the mechanisms underlying LMO4-mediated proliferation in response to ErbB2, we evaluated the effects of LMO4 knock-down on Cyclin D1, an ErbB2 target that is essential for formation of ErbB2-induced mammary tumors in mice (Lee et al., 2000) and a well-established modulator of the G1/S cell cycle transition (Resnitzky et al., 1994). RNAi was used to knock-down LMO4 in BT-474 cells and Cyclin D1 protein expression was analyzed by immunoblotting. Silencing LMO4 protein by ∼90% resulted in ∼80% reduction in Cyclin D1 protein levels as well as decreased expression of another regulator of the G1/S transition, Cyclin E (Figure 4a/b). Importantly, loss of LMO4 resulted in decreased Cyclin D1 expression in several cell lines, including the non-ErbB2 overexpressing cell line, HEK-293T (data not shown), and the ErbB2-overexpressing cell line SKBR3 (Figure 4a), indicating that LMO4 is necessary for sustained Cyclin D1 expression in multiple cell types, including those that do not require ErbB2 for proliferation. Because LMO4 is a transcriptional modulator, we analyzed *Cyclin D1* mRNA by real time RT-PCR. Similar to the changes in protein expression, inhibition of *LMO4* expression using two different siRNAs resulted in a concomitant decrease in *Cyclin D1* mRNA (Figure 4c). Over the course of several RNAi experiments, we achieved titratable suppression of LMO4 resulting in an ability to assess the dose-response relationship between LMO4 and Cyclin D1. There is a significant linear relationship between *LMO4* and *Cyclin D1* mRNAs, particularly in the lower range of *LMO4* mRNA levels ($r = 0.93$, Figure 4d). Taken together, these data suggest that LMO4 regulates the expression of Cyclin D1.

The experiments performed above utilized BT-474 cells, in which ErbB2 is constitutively active, and do not address the potential role of LMO4 as an intermediary between acute ErbB2 activation and upregulation of Cyclin D1. To determine if LMO4 is essential for transient induction of Cyclin D1 by active ErbB2 signaling, we used a ligand activation model. This involved treating MCF-7 breast cancer cells, which express low levels of ErbB2, with heregulin, a ligand for ErbB3 and ErbB4 receptors (Stern, 2003) that indirectly activates ErbB2 by heterodimerization (Beerli et al., 1996) and accelerates cell cycle progression (Lewis et al., 1996). Heregulin induced Cyclin D1 protein levels by 3 fold and this induction was blocked with LMO4 silencing (Figure 4e/f). Hence, LMO4 is necessary for the acute upregulation of Cyclin D1 that occurs with ErbB family signaling. The critical role that Cyclin D1 plays in the cell cycle and the exquisite reliance of Cyclin D1 on LMO4 suggests that LMO4 may be an obligate intermediate between ErbB2 and cell cycle progression.

LMO4 regulates G2/M transition by inducing cullin-3 expression

The requirement for LMO4 expression for proliferation and sustained expression of cell cycle genes involved in the G1/S transition, such as Cyclin D1 and Cyclin E, suggested that LMO4 may be necessary for traversing the G1/S transition in ErbB2-dependent breast cancer cells. To test this supposition, LMO4 expression was silenced in BT-474 and SKBR3 cells and cell cycle progression analyzed by flow cytometry. Knock-down of LMO4 had no significant effect in the percentage of cells in subG1, indicating that loss of LMO4 does not result in an increase in cell death in these cells (Figure 5a/b). Unexpectedly, when LMO4 expression was silenced, the percentage of cells in G1 decreased while the percentage of cells in G2/M increased. In BT-474 cells, reducing LMO4 expression resulted in a 30% increase in the percentage of cells in G2/M (Figure 5a). In SKBR3 cells, LMO4 knock-down resulted in 70% increase in the percentage of cells in S phase and 50% increase in the percentage of cells in G2/M, when compared to non-silencing controls (Figure 5b). Thus, LMO4 loss induces a G2/M phase arrest in both cell lines, and it also induces an S phase delay in SKBR3 cells. The S phase delay could be caused by additional activation of cell cycle checkpoints involved in detection of DNA damage (Ismail et al., 2007). LMO4 silencing was previously reported to cause a G1/S arrest (Sum et al., 2005b). However, those studies contrast with the current analysis by utilizing breast cancer cells that were not dependent on ErbB2 signaling. It is feasible that LMO4 integrates multiple signals that promote cell cycle progression and that it regulates target genes in a breast cancer subtype-specific manner. We suggest that LMO4 directly targets G2/M regulators and this leads to the indirect regulation of G1-associated cell cycle modulators (*i.e.*, Cyclins D1 and E).

To further examine the mechanism by which LMO4 controls proliferation, we sought to identify cell cycle genes that are more proximally regulated by LMO4. To detect these primary changes, we chose the earliest time-point after siRNA transfection that results in >80% suppression of LMO4. Changes in mRNA expression were then assessed using a qRT-PCR array that interrogates 84 cell cycle genes. Using a 2-fold change and statistical significance of $p<0.05$ as thresholds, only a single gene was differentially expressed. The mRNA for *cullin-3* [*cul3*] was decreased ∼25 fold immediately following LMO4 silencing ($p<0.02$, compared to non-silencing controls, Figure 5c). The change in *cul3* was confirmed by semi-quantitative RT-PCR of independent knock-down experiments (Figure 5d). Cul3-based E3 ubiquitin ligases are multiprotein complexes that regulate the turnover of proteins involved in cell division and assembly of the mitotic spindle. These complexes also sense DNA damage (Ribar et al., 2007). Cullin-3 acts as a scaffold for RING domain proteins that recruit the ubiquitin conjugating enzyme (E2) and substrates with recognition modules known as BTB [Broad-complex, Tramtrack, Bric-a-brac], bringing them into close proximity to the ubiquitin ligase complex (E3) (Xu et al., 2003). Transient knock-down of cul3 in HeLa cells results in misaligned chromosomes during metaphase, delayed entry into anaphase, and failure to complete cytokinesis (Sumara et al., 2007), indicating that cul3 is essential for mitotic progression. The role of cul3 during mitosis and the dependency of cul3 expression on LMO4 suggested that LMO4 stimulates cell cycle progression by inducing expression of cul3. Since LMO4 does not directly bind DNA, determining if LMO4 directly regulates the *cul3* gene will first require identification of a *cis*-acting LMO4 response

element(s) within the gene followed by characterization of LMO4's interaction with proteins that bind such element(s).

Since LMO4 is itself regulated by ErbB2 signaling, we determined if ErbB2 activity is also necessary for *cul3* expression. Treatment of BT-474 cells with the ErbB2 inhibitor, AG825, results in a decrease of *LMO4* mRNA (Figure 2c) and this is accompanied by a ∼50% reduction in *cul3* mRNA (Figure 5e). The requirement for LMO4 for ErbB2-induced G2/M progression and the dependency of *cul3* on sustained LMO4 expression lead us to conclude that LMO4 is an intermediary between ErbB2 signaling and cul3-induced progression through mitosis.

LMO4 expression oscillates throughout the cell cycle with peak expression occurring at G2/M phase

The 3' UTR of the *LMO4* mRNA contains three adenine- and uridine-rich elements [ARE] that are involved in regulation of *LMO4* mRNA stability in response to fluctuating levels of extracellular ATP (Chen et al., 2007). AREs also promote the rapid decay of many cell cycle regulating mRNAs, including *Cyclin D1*, *Cyclin B1*, *Cyclin A* and *p21* (Lal et al., 2004; Wang et al., 2000). The ability of LMO4 to control proliferation coupled with the fact that the *LMO4* mRNA can undergo rapid decay lead us to hypothesize that LMO4 protein expression may oscillate throughout the cell cycle. To test this, we quantified the pattern of LMO4 expression in synchronized HEK-293T cells as they progressed through the cell cycle following release from an aphidicolin-induced G1/S blockade. HEK-293T cells were used since they are resistant to chromosomal breaks and gaps after aphidicolin treatment (Caporossi et al., 1995; Junaid et al., 2007). In addition, LMO4 silencing inhibits growth of these cells (data not shown). LMO4 protein expression increased ∼3 fold nine hrs after aphidicolin release compared to earlier time points, and then decreased back to baseline (Figure 6b), suggesting LMO4 expression is dynamic and that it fluctuates during cell division. Peak LMO4 expression coincides with maximal accumulation of cells in the G2/M phase of the cell cycle, while the lowest LMO4 levels occurred during G1 (Figure 6b). The changes in LMO4 expression preceded oscillations in *cul3* mRNA (Figure 6b). Maximum expression of *cul3* occurred during late G2/M phases (Figure 6b), which coincide with its role in mitosis and cytokinesis. The peak in LMO4 protein precedes the peak in *cul3* mRNA expression, which is consistent with a role for LMO4 in regulating *cullin-3* and progression through the cell cycle. Of note, the pattern of LMO4 expression during the cell cycle is reminiscent of Cyclin B1 expression (Pines et al., 1989), opening the possibility that LMO4 may be regulated by this cyclin. Moreover, the modulation of LMO4 in these kidney derived cells suggests that LMO4 may be an important regulator of the cell cycle in many cell types. Together, these results indicate that LMO4 acts as a classic cell cycle regulator, being modulated by specific stages of the cycle and having a distinct target (*i.e.*, *cul3*) that is essential for traversing G2/M phase.

In summary, we have found that LMO4 is an essential target of ErbB2-induced cell cycle progression. As depicted in Figure 7, our studies revealed that *LMO4* gene expression is induced by ErbB2 and PI3K signaling and that LMO4 is required for progression through the cell cycle since loss of LMO4 results in a G2/M arrest. Early events occurring after

LMO4 silencing suggest the ubiquitin E3 ligase scaffold protein, cullin-3, is a target of LMO4 that promotes progression through G2/M. The arrest of cells at G2/M following loss of LMO4 inhibits subsequent progression through the cell cycle and thus also impedes the accumulation of the G1 cyclins. Furthermore, LMO4 displays a periodicity reminiscent of many other integral cell cycle regulatory proteins.

The role of cell cycle proteins in human breast cancers is indubitable. Proliferation rates and, more specifically, expression of cell cycle-correlated genes predict outcome of breast cancer, regardless of initiating oncogenic event (Mosley et al., 2008). In keeping with its essential role in proliferation, evaluation of publicly available gene expression data indicates that the highest quartile of *LMO4* mRNA expression is associated with poorly differentiated, high grade breast tumors when compared to well-differentiated and low-grade tumors in two independent studies (Table 2) (Rhodes et al., 2004; Ivshina et al., 2006; Desmedt et al., 2007). Given LMO4's newly demonstrated role in proliferation, it is not surprising to observe that high nuclear LMO4 expression correlates with poor survival of breast cancer patients (Sum et al., 2005b). Thus, we conclude that LMO4 functions as a general cell cycle regulator that is commandeered by ErbB2 to drive mitotic progression by regulating expression of various cell cycle proteins.

Materials and Methods

Cells

BT-474 (ATCC, Manassas, VA, USA) were grown in Hybricare 46X media (ATCC) supplemented with 0.01 mg/mL bovine insulin and 10% serum. MCF-7 and HEK-293T cells (ATCC) were grown in DMEM (Mediatech, Manassas, VA, USA) with 10% FBS. SKBR3 (ATCC) were grown in McCoy's medium with 10% FBS (Mediatech).

Compounds and expression vectors

Following 24 hrs of serum-starvation, heregulin (377-HB, 5 ng/mL, R&D systems, Minneapolis, MN, USA) or the following inhibitors were used: Tyrphostin AG825 (ErbB2, 20 μM, Calbiochem, San Diego, CA, USA), ZD1836 (EGFR, 1 μM, AstraZeneca, Wilmington, DE, USA), LY294002 (PI3K, 40 μM, Promega, Madison, WI, USA), PD98059 (MEK, 20 μM, Promega), JNK Inhibitor II (JNK, 25 μM, Calbiochem) and AKT Inhibitor V (AKT, 5 μM, Calbiochem). Myristolated-AKT (constitutively active AKT) and AKT K179M (kinase inactive AKT) vectors were a gift from S. Brady-Kalnay. LZRS-vector and LZRS-ErbB2 plasmids were a gift from E. Johnson and G. Bernardo.

BrdU Immunostaining

After siRNA transfection, cells were pulsed with 100 μM BrdU (Sigma, St. Louis, MO, USA) and fixed with methanol. Cells were then incubated with a BrdU antibody (BD Biosciences, San Jose, CA, USA) following the manufacturer's protocol. FITC-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) was used for detection and vectashield aqueous mounting media with DAPI was used as counterstain. At least 3 independent experiments were performed, each with at least two replicate samples. Percent of BrdU-positive cells was calculated by obtaining images of at least 5 different

fields/replicate and counting the number of BrdU-positive cells and the number of total cells/field. In this manner, a minimum of 200 total cells/replicate sample were counted.

Cell cycle arrest

HEK-293T cells were synchronized with 1.5 μg/mL of aphidicolin (Sigma) in serum-free media. After 14 hrs, medium was changed to media containing 10% serum. Cells were then harvested at various time points for protein, mRNA and flow cytometry analysis.

Flow cytometry

Cells were harvested and fixed with 0.25% methanol-free formaldehyde for 10 min at 37°C. Fixed cells were resuspended in Propidium Iodide/RNase solution (100 μg/mL propidium iodide, 0.1% Nonidet P-40, 0.1% NaN_3 and 1.2% RNase A) and incubated for 30 min at 37°C. Flow cytometry was completed by the Case Comprehensive Cancer Center Cytometry Core facility.

Statistical Analysis

Statistical analyses were performed using one or two-tailed Student's t-test with p-values less than 0.05 considered significant. Correlation coefficient was determined by calculating the Pearson product moment correlation coefficient, r.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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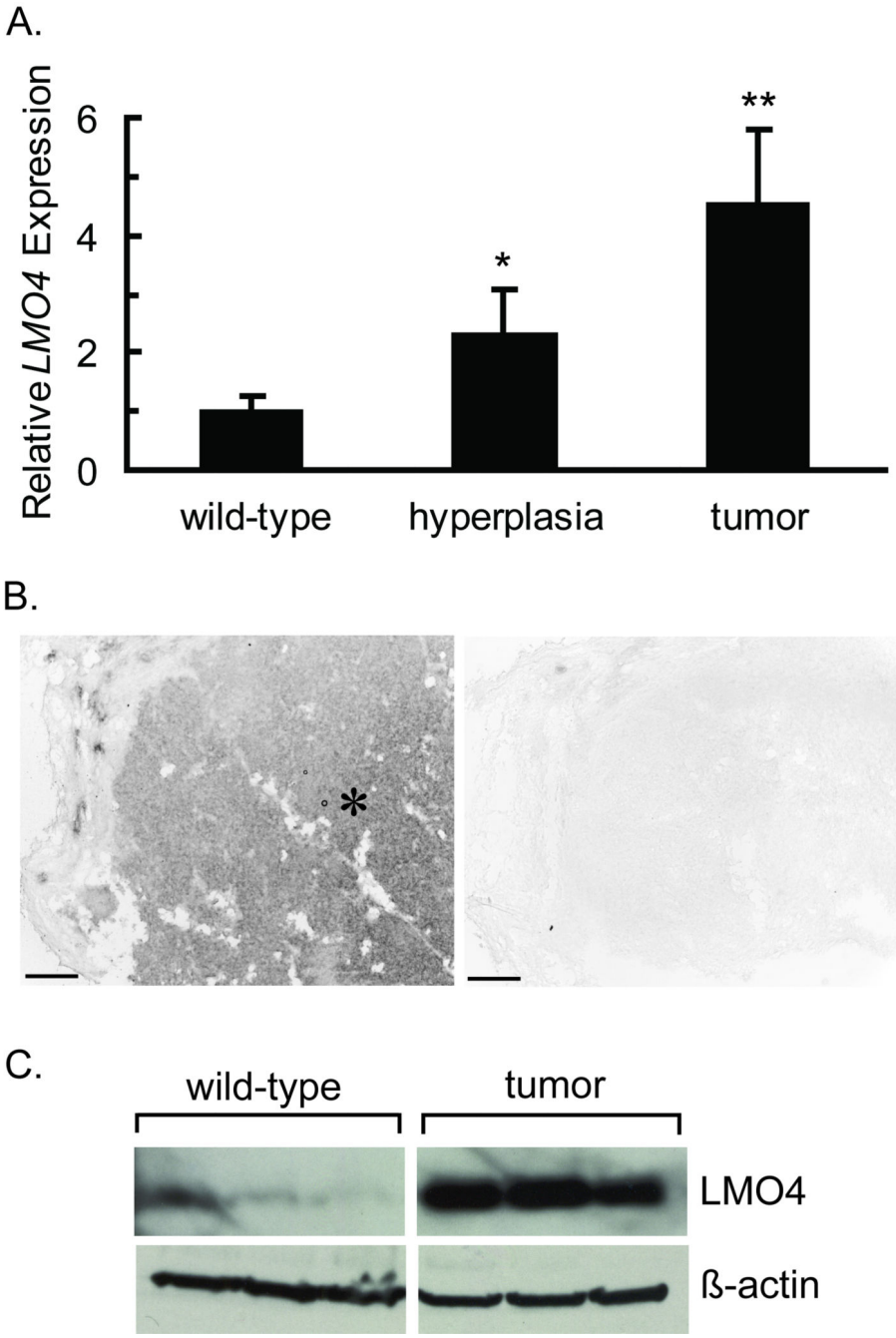

Figure 1.

LMO4 mRNA and protein are increased in ErbB2-induced tumors. (**a**) Tumor (n=5) and hyperplastic (n=4) mammary glands from MMTV-c-*Neu* mice were compared to wild-type mammary glands (3 pools of n=5) using gene expression microarrays. *LMO4* mRNA was elevated 4.5 fold in tumors and 2.3 fold in hyperplastic glands when compared to wild-type glands. * $p<0.05$, ** $p<0.005$, respectively. (**b**) An antisense LMO4 probe was used for *in situ* hybridization of an MMTV-c-*Neu* tumor. Asterisk (*) indicates tumor tissue. Sense control probe hybridized to the same tumor is shown to the right. (**c**) Whole tissue lysates of

mammary glands from age-matched wild type mice (n=3) and tumors (n=3) from MMTV-c-*Neu* mice were analyzed by western blotting. β-actin was used as loading control.

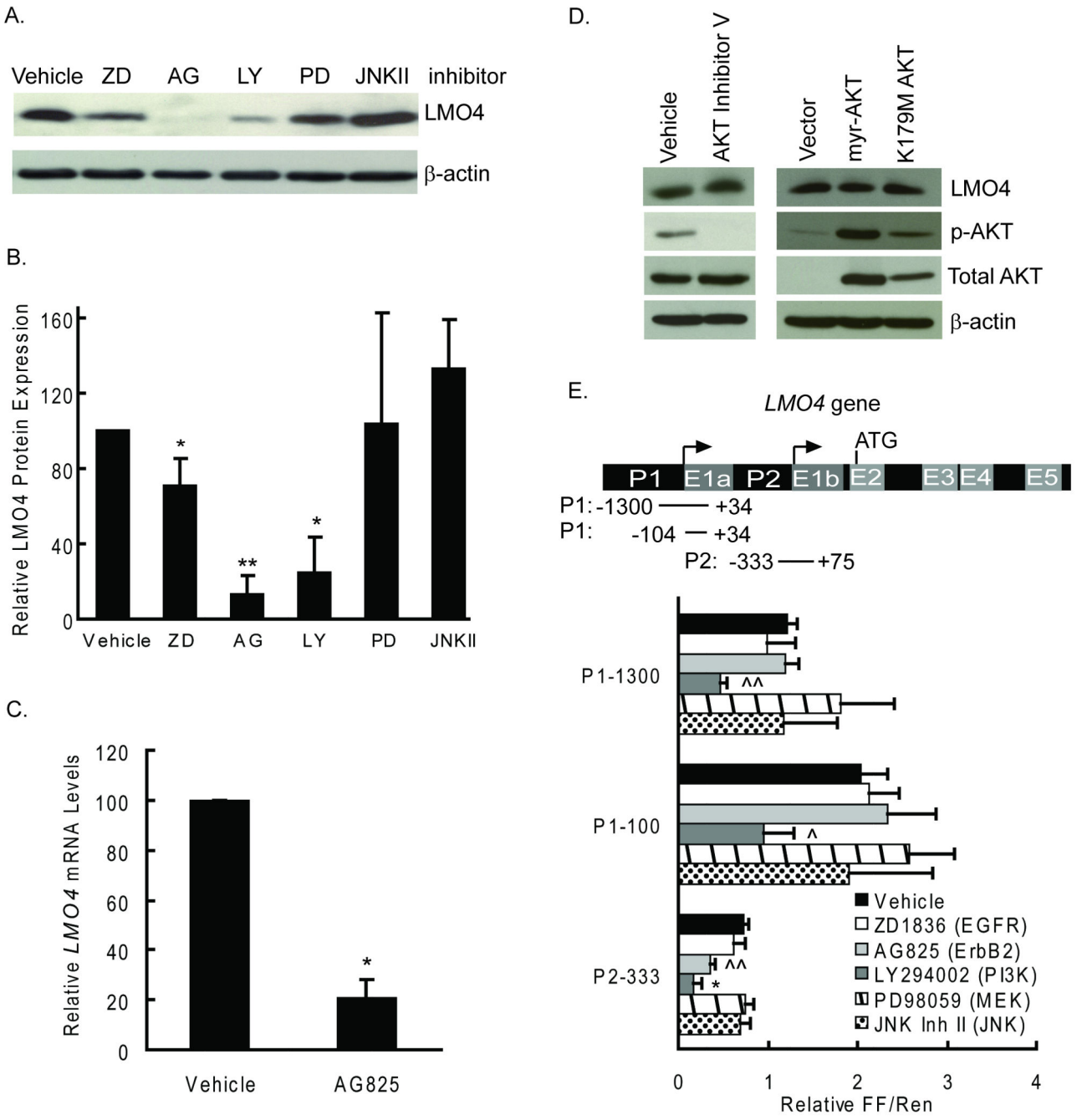

Figure 2.

ErbB2 and PI3K activity are necessary for LMO4 expression. (**a**) Western blot analysis of LMO4 expression in BT-474 cells treated with the following small molecule inhibitors: ZD1836 (EGFR, 1 μM), AG825 (ErbB2, 20 μM), LY294002 (PI3K, 40 μM), PD98059 (MEK, 20 μM), and JNK Inhibitor II (JNK, 25 μM). β-actin was used as a loading control. (**b**) Quantification of (a). LMO4 was expressed relative to β-actin. Values are the average of at least 4 independent experiments. Error bars depict standard deviations. * $p<0.05$ and ** $p<5\times10^{-4}$ compared to vehicle. (**c**) Total RNA was isolated 12 hrs after treatment of BT-474

cells with AG825 (ErbB2, 20 μM) and analyzed for *LMO4* mRNA expression using real time RT-PCR. Values represent the averages of at least 4 experiments. * $p<5.0 \times 10^{-7}$ compared to vehicle-treated cells. (**d**) (left panel) BT-474 cells were treated with vehicle or AKT Inhibitor V (AKT, 5 μM) for 24 hrs and then cell lysates were collected for immunoblotting against LMO4. phospho-AKT (pAKT) was used to demonstrate inhibitor efficacy while total AKT and β-actin were used as loading controls. (right panel) HEK-293T cells were transiently transfected with 1.5 μg of myristolated AKT (myr-AKT), kinase inactive AKT (K179M AKT) or empty vector. Cells were lysed after 48 hrs and analyzed for LMO4 protein expression. p-AKT and total AKT levels are shown to confirm plasmid activity. β-actin was used as loading control. (**e**) (upper panel) Schematic of the *LMO4* gene which has 5 exons (E1-E5) and two promoters (P1 and P2). The alternative transcription start sites yield identical protein products because the translation start site (ATG) is in Exon 2. The 5' and 3' ends of each construct are indicated below each promoter. (bottom panel) Activity from two proximal *LMO4* promoter constructs (P1) and one distal *LMO4* promoter construct (P2) was assessed in BT-474 cells treated with various inhibitors. All luciferase values were first normalized to a renilla transfection efficiency control and then expressed relative to values obtained with a minimal SV40 promoter. * $p<5\times10^{-4}$, ^ $p<0.05$, ^^ $p<0.001$.

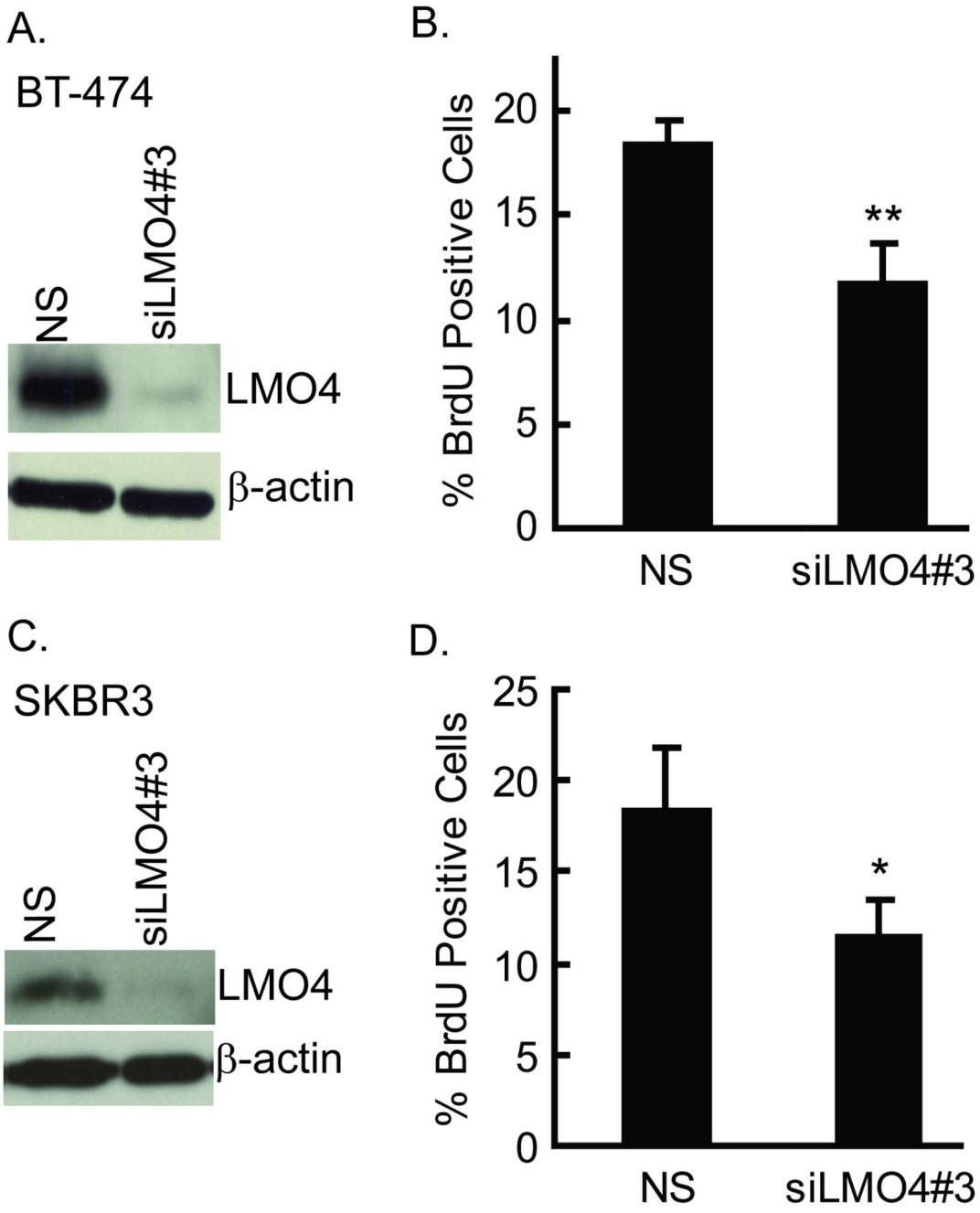

Figure 3.

LMO4 regulates proliferation of ErbB2-dependent breast cancer cells. BT-474 and SKBR3 cells were transiently transfected with non-silencing siRNA (NS), or an siRNA directed to LMO4 (siLMO4#3). 36 hrs after transfection, cells were pulsed with BrdU for 1 hr. The percentage of BrdU positive cells was determined by immunofluorescence. (**a,c**) Immunoblotting was performed to confirm knock-down of LMO4 in BT-474 cells (a) and SKBR3 cells (c). β-actin was used as loading control. (**b,d**) Quantitation of proliferation rate was performed by calculating the percentage of BrdU-positive cells in a minimum of three

independent experiments. Values are means ± standard deviations. * $p<0.05$, ** $p<0.01$, compared to non-silencing siRNA control.

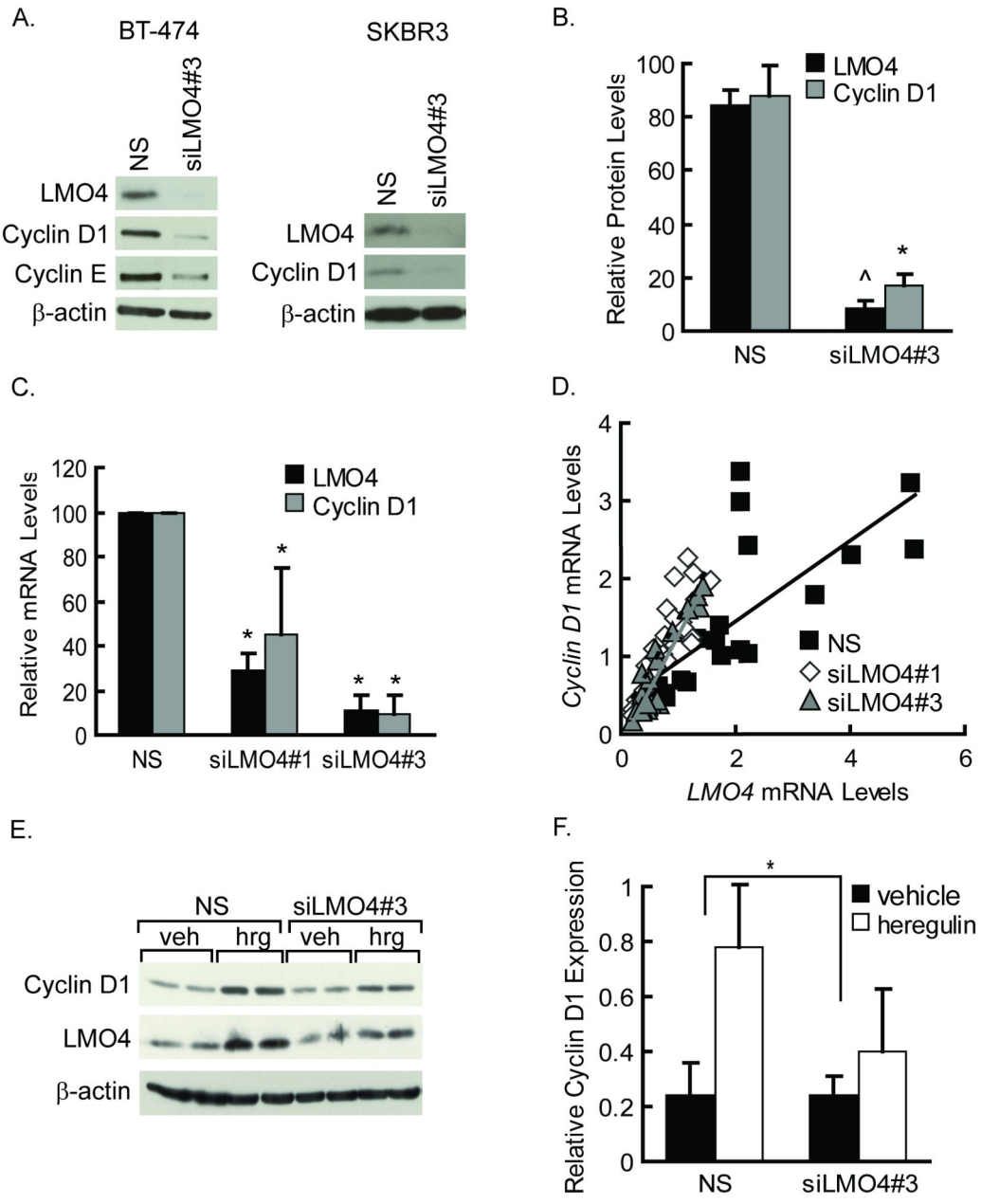

Figure 4.

LMO4 is required for maintenance of *Cyclin D1* mRNA and protein expression in cells with constitutive ErbB2 signaling as well as for heregulin-induced upregulation of Cyclin D1. (**a,b**) Analysis of changes in Cyclin D1 and Cyclin E protein levels in response to LMO4 silencing. (**a**) BT-474 (left panel) and SKBR3 (right panel) cells were transiently transfected with non-silencing siRNA (NS) or siRNA directed to LMO4 (siLMO4#3) and whole cell lysates were collected 36 hrs post-transfection. Representative western blots for Cyclin D1 and Cyclin E are shown. β-actin was used as a loading control. (**b**) Quantitation of three independent experiments performed in BT-474 cells, each with at least 2 independent replicates. Values are means ± standard deviation. ˆ $p<5\times10^{-5}$, * $p<0.001$. (**c,d**) Analysis of

changes in *Cyclin D1* mRNA expression in response to changes in *LMO4*. RNA was isolated 24 hrs after transfection of BT-474 cells with non-silencing siRNA (NS), or two different siRNA directed to LMO4 (siLMO4#1 and siLMO4#3), and analyzed by real time RT-PCR. Values were expressed relative to GAPDH and normalized to non-silencing control. (**c**) Bars represent means ± standard deviations from 3 separate experiments each with 6 independent replicates. * $p<0.05$ (**d**) Scatter plot analysis of representative experiments from (c) including linear regressions. The correlation coefficient between *Cyclin D1* mRNA and *LMO4* mRNA is 0.93. (**e,f**) MCF-7 cells were incubated for 24 hrs with either 5 ng/mL of heregulin (hrg) or vehicle following transient transfection with an LMO4 siRNA (siLMO4#3) or a non-silencing siRNA (NS) control. (**e**) Representative western blot (**f**) Bars represent the means ± standard deviations of at least 3 independent experiments. The Cyclin D1 fold induction between NS and siLMO4#3 treated cells was significantly different (* $p<0.05$).

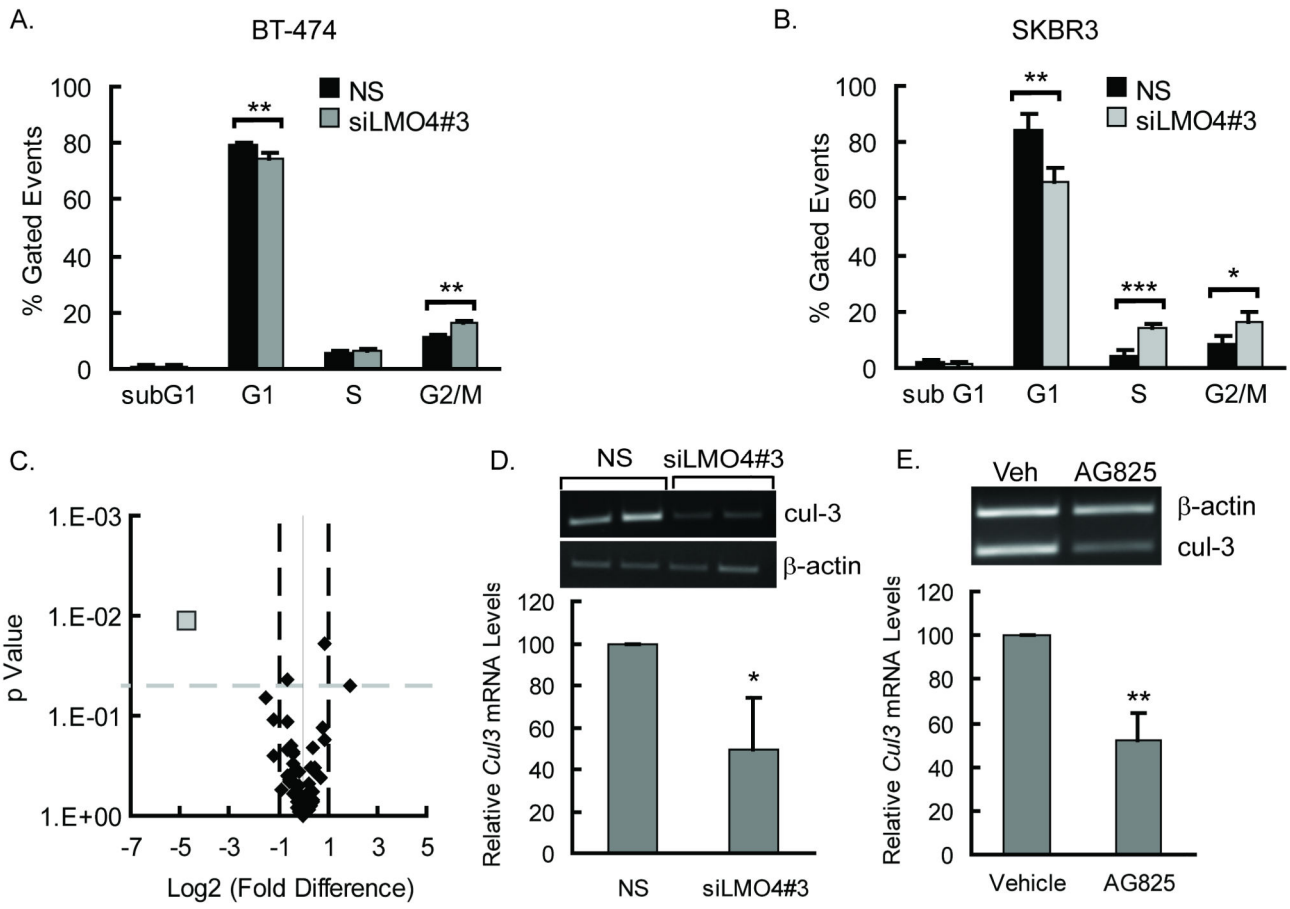

Figure 5.

LMO4 regulates G2/M transition by inducing cullin-3 expression. (**a,b**) BT-474 (a) and SKBR3 (b) cells were transfected with a non-silencing (NS) siRNA or siRNA directed to LMO4 (siLMO4#3). Thirty-six hours after the end of transfection, cells were stained with propidium iodide and examined by FACS. The graphs depict the percent of cells in subG1, G1, S and G2/M stages of the cell cycle. * $p<0.05$, ** $p<0.01$, *** $p<0.005$, compared to non-silencing control. (**c**) BT-474 cells were harvested 12 hrs after RNAi transfection. Suppression of LMO4 protein was confirmed and mRNA samples were analyzed by an RT-PCR array of 84 cell cycle genes. The graph plots the average fold change (Log2)*vs.* p-values for all 84 genes from 3 independent experiments, each with two replicate samples. Genes located outside the vertical, dashed black lines are changed more than 2 fold. Values above the horizontal, dashed grey line are statistically significant at $p<0.05$, compared to non-silencing control. ▪ represents the *cullin-3* mRNA (downregulated by 25 fold, $p<0.02$) (**d**) Semi-quantitative RT-PCR was used to confirm *cullin-3* (*cul3*) mRNA downregulation after silencing LMO4 in three additional experiments. β-actin was used as an internal control. (upper panel) Representative gel. (lower panel) Average *cul3* mRNA expression relative to β-actin ± standard deviation. * $p<0.05$. (**e**) BT-474 cells were treated with vehicle or AG825 (20 μM) for 12 hrs. RNA was isolated and analyzed by semi-quantitative RT-PCR for *cullin-3* (*cul3*) and *β-actin* mRNA expression (upper panel) Representative gel. (lower

panel) Bars are the mean *cul3* mRNA levels relative to *β-actin* mRNA ± standard deviations in 4 independent experiments. ** $p<0.005$, compared to vehicle control.

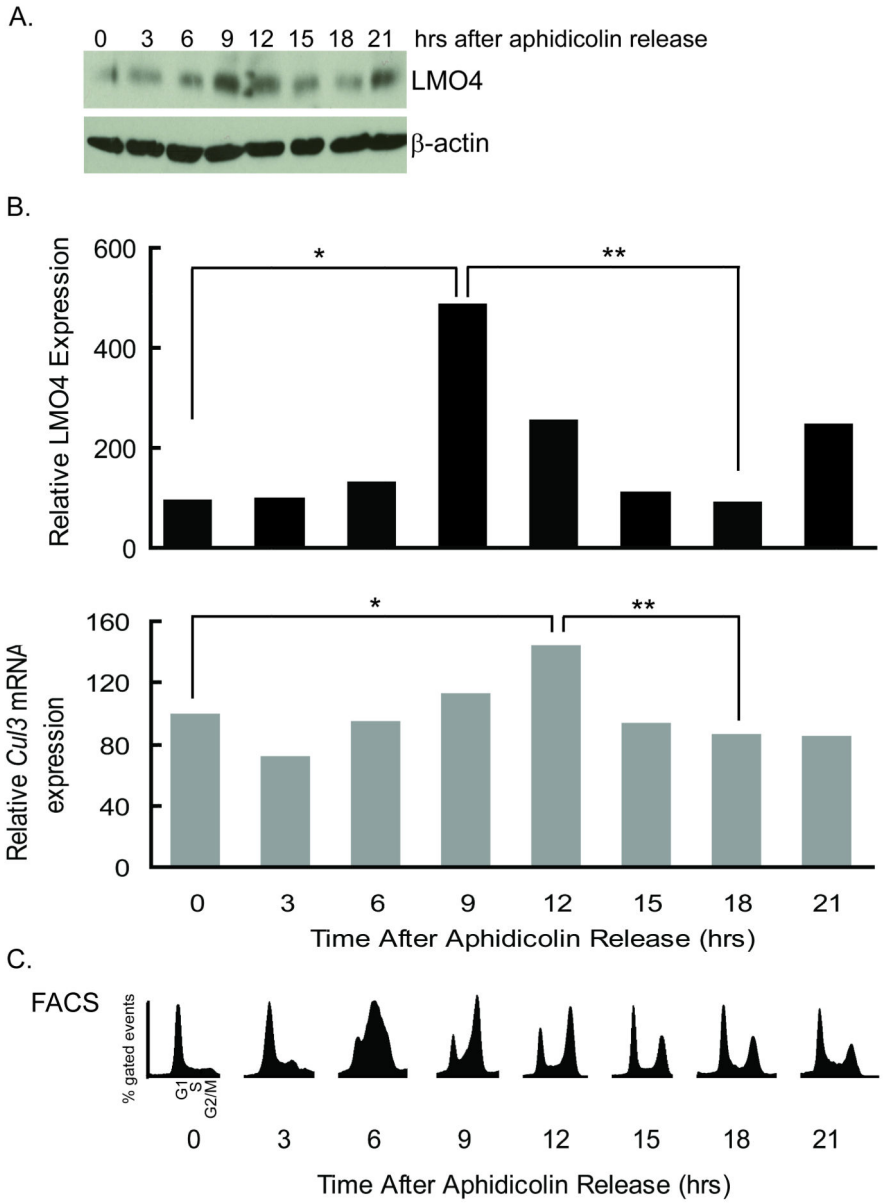

Figure 6.

LMO4 expression oscillates throughout the cell cycle with peak expression occurring at G2/M phase. HEK-293T cells were synchronized using aphidicolin (1.5 μg/mL). After re-initiation of the cell cycle with removal of aphidicolin and addition of serum, cells were harvested at the indicated times for western blotting and flow cytometry. (**a**) Representative western blot showing LMO4 protein levels at various time points after aphidicolin release. β-actin was used as loading control. (**b**) Quantitation of LMO4 protein expression (upper panel) and *cul3* mRNA expression (lower panel) from a representative experiment. LMO4 values were normalized to β-actin. *Cul3* mRNA levels were determined using real-time RT-PCR and were normalized to GAPDH. These experiments were repeated at least three times with similar results. * $p<0.05$ compared to baseline values, ** $p<0.05$ compared to values

after completing one cell division. (**c**) Flow cytometry analysis of propidium iodide-stained cells for each of the indicated time points shows progression through one complete cell cycle. Areas representing cells at G1, S and G2 phases of the cell cycle have been annotated.

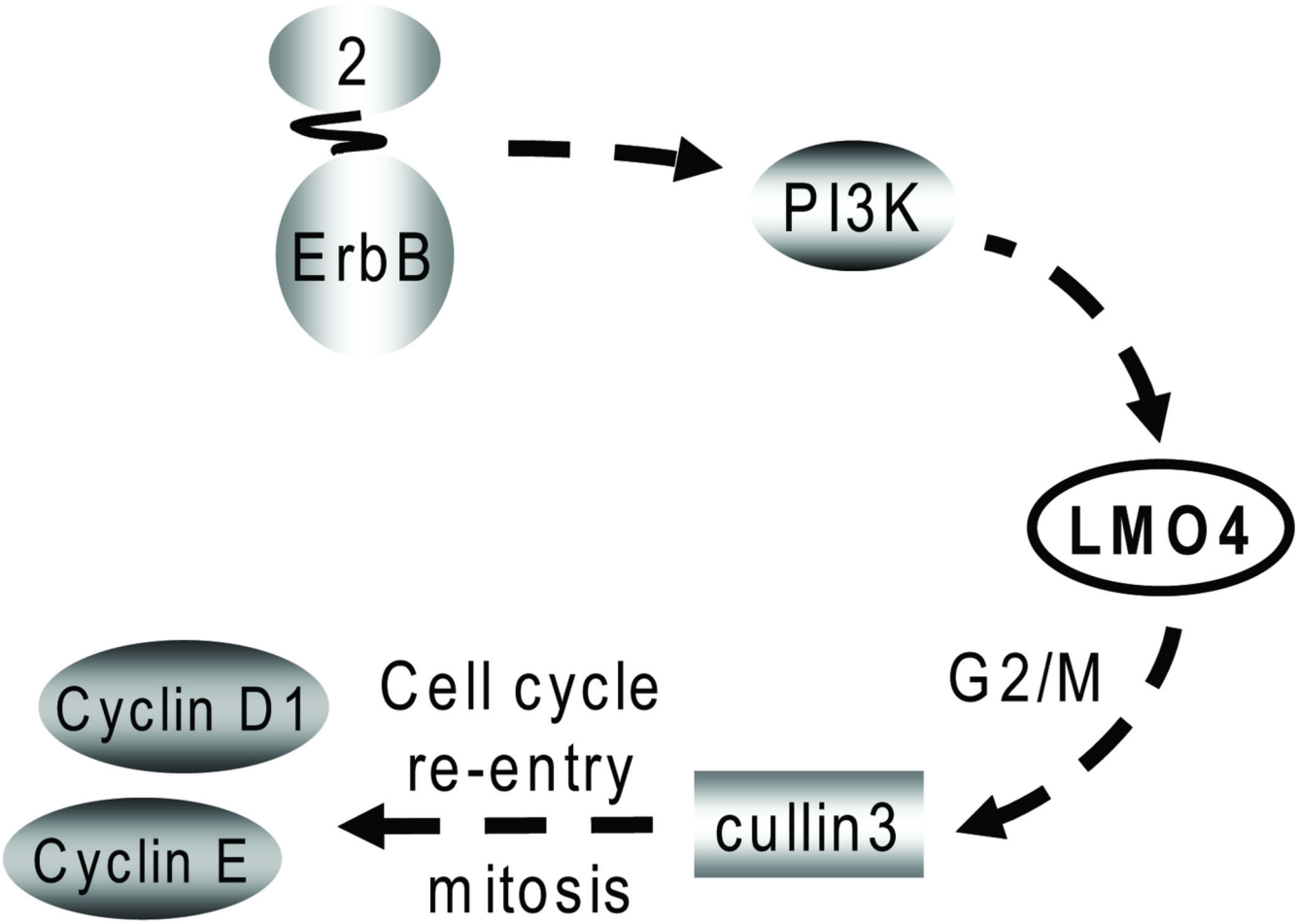

Figure 7.

LMO4 is an essential intermediate of ErbB2-induced proliferation. LMO4 is target of ErbB2 signaling that induces proliferation. This pathway begins with ErbB2 regulation of PI3K activity, which controls expression of the transcriptional modulator LMO4 and ultimately leads to induction of *cullin-3* gene expression. Cullin-3 in turn modulates cell cycle progression through G2/M. By regulating progression through G2/M, LMO4 also indirectly regulates expression of the G1 cyclins, Cyclin D1 and Cyclin E.

Table 1

LMO4 mRNA expression in murine models of breast cancer.

	Mouse Model						
	C(3) T-antigen	**WAP- T antigen**	**MMTV-c- myc**	**MMTV-Neu**	**MMTV-HA-ras**	**MMTV-PyMT**	**K4-Cre APC$^{cko/+}$**
LMO4 mRNA expression	n.c.	n.c.	n.c.	+	++	+	n.c.

Expression profiles of mammary gland tumors from mice overexpressing T-antigen, myc, ErbB2/Neu, HA-ras and polyoma middle T antigen (*PyMT*) (Desai, et.al., 2002) and mammary gland tumors from mice with targeted deletion of Adenomatous Poliposis Coli (APC), which results in aberrant Wnt signaling (Kuraguchi, et.al., 2009), were compared to normal mammary glands. Results for *LMO4* mRNA levels are shown above: (n.c.) no change, (+) increased levels and (++) highly increased levels, relative to control glands.

Table 2

LMO4 overexpression is associated with high-grade, poorly differentiated breast cancers.

Clinical parameter (Ivshina, et.al., 2006)	First Quartile Low *LMO4* expression # Tumors (%)	Fourth Quartile High *LMO4* expression # Tumors (%)	p-value
Elston Grade 1	21 (67.7)	7 (22.6)	
Elston Grade 3	10 (32.3)	24 (77.4)	$p < 5\times10^{-4}$
Clinical parameter (Desmedt, et.al, 2007)	First Quartile Low *LMO4* expression # Tumors (%)	Fourth Quartile High *LMO4* expression # Tumors (%)	p-value
Well differentiated	13 (46.4)	0 (0)	
Poorly differentiated	15 (53.6)	28 (100)	$p < 5\times10^{-5}$

Two publicly available cohorts of human breast tumors were ranked according to *LMO4* mRNA expression. The number of tumors corresponding to each clinical parameter was then evaluated for the first and fourth quartile fractions of *LMO4* expression.