

MUC1 regulates nuclear localization and function of the epidermal growth factor receptor

Benjamin G. Bitler¹, Aarthi Goverdhan² and Joyce A. Schroeder^{1,2,3,*}

¹Arizona Cancer Center, ²Department of Molecular and Cellular Biology, ³Bio5 Institute, University of Arizona, Tucson, AZ 85724, USA

*Author for correspondence (jschroeder@azcc.arizona.edu)

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Summary

Alteration of protein trafficking and localization is associated with several diseases, including cystic fibrosis, breast cancer, colorectal cancer, leukemia and diabetes. Specifically, aberrant nuclear localization of the epidermal growth factor receptor (EGFR), a receptor tyrosine kinase, is a poor prognostic indicator in several epithelial carcinomas. It is now appreciated that in addition to signaling from the plasma membrane, EGFR also trafficks to the nucleus, and can directly bind the promoter regions of genes encoding cyclin D1 (*CCND1*) and B-Myb (*MYBL2*). We have previously established that loss of MUC1 in an EGFR-dependent transgenic mouse model of breast cancer correlates with the loss of cyclin D1 expression. Here, we provide evidence for a novel regulatory function of MUC1 in the trafficking and nuclear activity of EGFR. We found that MUC1 and EGFR interact in the nucleus of breast cancer cells, which promotes the accumulation of chromatin-bound EGFR. Additionally, the presence of MUC1 results in significant colocalization of EGFR and phosphorylated RNA polymerase II, indicating that MUC1 influences the association of EGFR with transcriptionally active promoter regions. Importantly, we found that the loss of MUC1 expression resulted in a decrease in the interaction between EGFR and the *CCND1* promoter, which translated to a significant decrease in cyclin D1 protein expression. This data offers insights into a novel regulatory mechanism of EGFR nuclear function and could have important implications for evaluating nuclear localization in cancer.

Key words: MUC1, EGFR, Cyclin D1, Nuclear translocation, Breast cancer

Introduction

Subcellular protein trafficking is a highly regulated process that is essential for maintaining normal cell function and viability. In fact, the mislocalization of proteins has a fundamental role in promoting diseases such as cystic fibrosis, diabetes and several types of cancer (for a review, see Davis et al., 2007). In cancer specifically, aberrant nuclear localization of receptor tyrosine kinases, such as, c-MET, ErbB1/EGFR, ErbB2, ErbB3, ErbB4, FGF and VEGF, results in increased transcription of genes that promote chemoresistance, proliferation and disease progression (Feng et al., 1999; Lin et al., 2001; Maher, 1996; Ni et al., 2001; Offterdinger et al., 2002; Pozner-Moulis et al., 2006; Wang et al., 2004). Notably, these effects are not due to increased signal transduction, but are instead due to the ability of these proteins to act as transcriptional cofactors (Wang et al., 2004). A number of these proteins contain nuclear localization signals (NLSs), including the erbB family of receptors (ErbB1–ErbB4), which share a highly conserved tripartite NLS (consensus sequence: [RK]{3}-x{2,3}-[RK]{3,4}-x{2,3}-RR) (Hsu and Hung, 2007). Although this NLS can drive nuclear translocation, the receptors typically remain localized to the plasma membrane in polarized epithelium.

The epidermal growth factor receptor (ErbB1/EGFR) is an example of a protein that has altered subcellular localization in cancer. In normal polarized epithelium, EGFR is targeted to the basolateral membrane by well-conserved localization sequences (KRTLRLQLQERELVEPLTPSGEAP) (He et al., 2002). Under these circumstances, ligand stimulation induces receptor dimerization, ubiquitylation, endocytosis and degradation in the lysosome, thereby limiting EGFR expression and signaling (for a review, see Le Roy and Wrana, 2005). However, in cancer cells, it

has recently been discovered that EGFR undergoes retrograde trafficking through the endoplasmic reticulum Sec61 translocon, which is followed by release into the cytosol (Liao and Carpenter, 2007). Following this release from the membrane, the tripartite nuclear NLS of EGFR interacts with importin- β 1 allowing it to be shuttled through the nuclear pore complex into the nucleus (Lo et al., 2006). Once inside the nucleus, it has been proposed that EGFR directly interacts with the endogenous promoter and initiates transcription of the cell cycle regulator *CCND1*, which encodes cyclin D1 (Lin et al., 2001). Finally, the CRM1 exportin is capable of facilitating the nuclear export of EGFR (Lo et al., 2006). Of note, the presence of EGFR in the nucleus of tumor cells correlates with a poor clinical outcome in breast, ovarian, oropharyngeal, esophageal squamous cell and epithelial carcinomas (Xia et al., 2008).

The heterodimeric transmembrane mucin MUC1 is also highly expressed in epithelial carcinomas and inhibits the ubiquitylation of EGFR, altering normal EGFR trafficking (Pochampalli et al., 2007b). In polarized ductal epithelium, MUC1 is constitutively internalized and recycled between the apical membrane and the Golgi, which enables it to maintain a high level of O-glycosylation (Litvinov and Hilken, 1993). The apical localization of MUC1 is regulated by a well-conserved targeting sequence in the juxtamembrane domain (CQC) (Pemberton et al., 1996). In non-polarized breast epithelium, MUC1 and EGFR interact at the plasma membrane, resulting in increased EGFR internalization, the loss of lysosomal degradation and an increase in EGFR recycling (Pochampalli et al., 2007b). In addition to its membrane localization, MUC1 is overexpressed and mislocalized to the cytosol and nucleus in about 90% of breast adenocarcinomas,

correlating with poor prognosis (Rahn et al., 2001; Schroeder et al., 2004; Schroeder et al., 2001; Zotter et al., 1988). Notably, the loss of MUC1 significantly delays tumor onset in an EGFR-dependent (WAP-TGF α) transgenic breast cancer model (Pochampalli et al., 2007a; Sandgren et al., 1995). In this study, loss of MUC1 within EGFR-dependent mammary gland tumors is also associated with a reduction of cyclin D1 expression (Pochampalli et al., 2007a).

MUC1 trafficking to the nucleus is due to the presence of a non-classical NLS (RRK) that interacts with nucleoporin-62 and importin- β 1, allowing for the nuclear co-translocation of numerous proteins, including γ -catenin, β -catenin and estrogen receptor (Leng et al., 2007; Li et al., 2003; Wei et al., 2006; Wen et al., 2003). In fact, the transcriptional activity of β -catenin is dependent on nuclear localization of MUC1, indicating that MUC1 is acting as co-factor (Huang et al., 2003; Leng et al., 2007). In addition, transcriptional targets of these proteins are activated in a MUC1-dependent manner. Given the influence of MUC1 on EGFR trafficking following receptor activation, and the newly described EGFR nuclear-trafficking pathway, our current study was designed to determine whether MUC1 functions as a regulator of EGFR nuclear translocation and transcriptional activation of *CCND1*. In this report, we demonstrate a role for MUC1 in EGFR regulation. Through biochemical and genetic analysis, we show that MUC1 alters the nuclear localization and function of EGFR in a manner that might have important consequences for EGFR-dependent tumors.

Results

MUC1 promotes accumulation of nuclear EGFR

The loss of MUC1 expression significantly suppresses EGFR-dependent cancer progression and cyclin D1 protein expression in the WAP-TGF α model (Pochampalli et al., 2007a). In the current study, we set out to determine whether the mechanism by which MUC1 alters EGFR-dependent cyclin D1 expression is via regulation of nuclear trafficking and direct transcriptional function of EGFR.

To examine the effect of MUC1 on EGFR cellular localization, we used two cell lines expressing MUC1 (MCF10A and MDA-MB-468). Cells were transfected with either *MUC1*-specific siRNA to knock down MUC1 protein expression or a control siRNA, and changes in EGFR localization were evaluated. Optimal knockdown of MUC1 was observed after treatment with either *MUC1*-1- or *MUC1*-2-specific siRNAs after 3 days (MCF10A), as we have previously demonstrated with these *MUC1* target sequences (Fig. 1) (Pochampalli et al., 2007b). For all fractionation studies, cells were serum-starved for 18 hours, treated with 10 ng/ml EGF on ice for 10 minutes (to saturate membrane-bound receptors), and then excess ligand was removed. Cells were then incubated at 37°C for 120 minutes to induce receptor endocytosis and trafficking. Cells were then fractionated by differential detergent fractionation, which takes advantage of the ionic properties of different detergents to isolate the membrane (Triton X-100), cytosolic (digitonin) and nuclear (Tween40) protein fractions (Ramsby et al., 1994). Note that the membrane fractions include plasma membrane, mitochondria, Golgi, endosomes and the endoplasmic reticulum (ER). Proteins isolated from the membrane, cytosolic and nuclear compartments were then separated by SDS-PAGE and examined by subsequent immunoblotting to determine the effects of MUC1 expression on EGFR localization. To confirm fraction purity and equal loading, expression of proteins known to localize in each

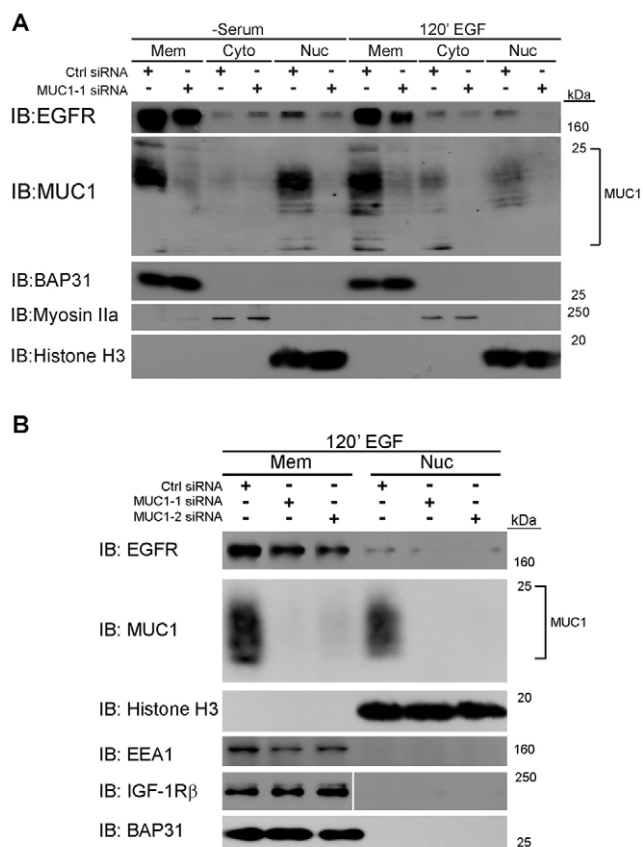


Fig. 1. MUC1 promotes nuclear localization of EGFR. (A) MDA-MB-468 breast cancer cells or (B) MCF10a immortalized breast epithelial cells treated for 3 days with control (Ctrl) or *MUC1* (*MUC1*-1 or *MUC1*-2) siRNA, were either serum starved (-serum) for 18 hours or serum starved and then treated with EGF (10 ng/ml, 120 minutes). Cells were fractionated and protein (6 μ g) was separated by SDS-PAGE. After transfer to PVDF, proteins were immunoblotted (IB) for EGFR (Santa Cruz, 1005) and MUC1 (Neomarkers, CT2). Fraction purity and loading were determined by immunoblotting for BAP31 (Affinity Bio-Reagents, MA3-002; ER Membrane, Mem), IGF-1R β (Santa Cruz, SC-713; plasma membrane), EEA1 (Santa Cruz, SC-33585; Endosomes), myosin IIa (Santa Cruz, D16; cytosol, Cyto), and histone H3 (Santa Cruz, C-16; Nucleus, Nuc). White lines through blots indicate same samples and exposure but were non-contiguous. Molecular masses (kDa) are indicated on the right. Note that the cytoplasmic domain of MUC1 runs as several species of 14-28 kDa, because of different glycosylation states (Schroeder et al., 2001).

cellular compartment were analyzed: plasma membrane, IGF-1R β ; ER membrane, BAP31; Endosomes, EEA1; cytosol, myosin IIa; nucleus, histone H3 (Ramsby et al., 1994).

Analysis of protein lysates from MDA-MB-468 cells revealed that MUC1 and EGFR were present in both the membrane and nuclear fractions under conditions of serum starvation and EGF treatment (Fig. 1A, top two panels). MUC1 expression did not affect EGFR localization to the membrane fraction in the absence of EGF, although loss of MUC1 resulted in decreased levels of EGFR in the membrane fraction in the presence of EGF. This recapitulates the result of a previous study that described increased EGFR membrane recycling in the presence of MUC1 (Fig. 1A, top panel) (Pochampalli et al., 2007b). The maximal nuclear EGFR accumulation was observed after 120 minutes of EGF stimulation,

a finding similar to that previously observed by another group (Liao and Carpenter, 2009). Interestingly, EGFR was observed in the nucleus in the presence or absence of EGF, indicating that nuclear EGFR trafficking can occur in the absence of serum in these cell lines (Fig. 1A, top panel). Importantly, in both serum-starved and EGF-treated cell lines, we observed that the absence of MUC1 inhibited the accumulation of EGFR in the nucleus (Fig. 1A, top panel). The effect of MUC1 on EGFR localization was found to be similar in MCF10A cells treated with either *MUC1-1* or *MUC1-2* siRNAs (Fig. 1B).

To determine whether this result was an artifact of ER or post-lysis contamination, we performed a high-salt nuclear protein extraction on cells treated with control or MUC1-1 siRNA. Cells were serum starved or stimulated with EGF for 0 or 120 minutes, subjected to high-salt protein extraction, and proteins were analyzed by immunoblotting. We discovered that after serum starvation without EGF treatment there was a minimal difference in total nuclear EGFR with respect to MUC1 expression. However, after 120 minutes of EGF treatment, there was a significant MUC1-dependent accumulation of total EGFR in the nucleus (supplementary material Fig. S1). This demonstrates that MUC1 promotes the accumulation of non-membrane-bound nuclear EGFR.

To visualize the subcellular localization of EGFR and MUC1, control and MUC1 siRNA-treated cells were analyzed by laser-scanning confocal microscopy. Cells were serum starved and either left untreated (Fig. 2A-H') or treated with 20 ng/ml EGF for 120 minutes (Fig. 2I-P'), then immunolocalized for MUC1 (Fig. 2A,E,I,M) and EGFR (Fig. 2B,F,J,N). We observed that in the absence of EGF, EGFR was mainly localized to the plasma membrane with limited cytosolic and nuclear localization, regardless of MUC1 expression (Fig. 2B,F). Under similar conditions, MUC1 localization was largely nuclear and cytosolic (Fig. 2A). Following EGF treatment, punctate staining of EGFR was observed within the cytosol of cells from both treatment conditions (Fig. 2J,N), but in MUC1 siRNA-treated cells, the cytosolic EGFR mainly localized to perinuclear regions (Fig. 2N,P', asterisks) whereas in control siRNA-treated cells that maintained MUC1 expression, significant punctate EGFR staining was

observed in the nucleus (Fig. 2J,L', arrowheads). These data corroborate our observation that MUC1 expression promotes nuclear accumulation of EGFR.

MUC1 and EGFR interact in the nucleus

We observed that the loss of MUC1 results in a significant decrease in levels of EGFR in the nucleus. We next wanted to determine whether MUC1 and EGFR were interacting within the nuclear compartment. MDA-MB-468 and MCF10A cells were treated as described in the previous section and subjected to differential detergent fractionation. To determine whether MUC1 and EGFR interacted within the nucleus of these cells, we isolated the nuclear protein (400 µg) and performed immunoprecipitation with EGFR and mouse IgG, followed by immunoblotting with antibodies against EGFR and MUC1. Following 120 minutes of EGF treatment at 37°C, MUC1 and EGFR formed a complex in the nucleus (Fig. 3A, top panel). Additionally, nuclear protein lysates were analyzed to confirm MUC1 expression and the resulting EGFR nuclear accumulation (Fig. 1B).

To determine whether interacting MUC1 and EGFR in the nucleus also bound nuclear DNA, we performed a fractionation that would allow us to distinguish between soluble and chromatin-bound nuclear proteins. After EGF treatment, cells were subjected to subcellular fractionation and the nuclear protein bound to chromatin was isolated as a separate fraction from the soluble nuclear protein. This was accomplished by first gently collecting protein from the nucleus and then collecting the protein-chromatin complexes. These complexes were treated with Micrococcal nuclease, which released chromatin-associated protein into the supernatant. We found that although EGFR was present at the same levels in the soluble nuclear fraction regardless of MUC1 expression, EGFR was only bound to chromatin in the presence of MUC1 (Fig. 3B, Nuc CB). These data demonstrate that the interaction of EGFR with chromatin is MUC1 dependent. Moreover, the results address the question of whether MUC1-dependent changes in EGFR levels in the nucleus are a result of altered EGFR degradation, because soluble nuclear EGFR was equivalent, regardless of MUC1 expression (Fig. 3B, Nuc S).

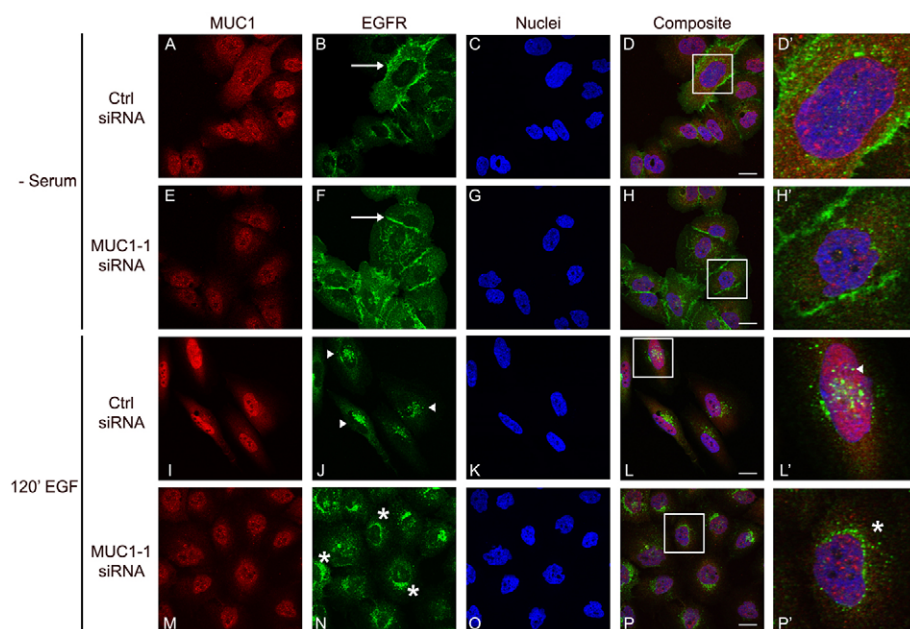


Fig. 2. MUC1 promotes nuclear EGFR accumulation. Immortalized breast epithelial cells (MCF10A) were treated with *MUC1-1* (E-H', M-P') or control (Ctrl) siRNA (A-D', I-L') and stimulated with EGF (20 ng/ml) for 120 minutes. (I-P') Cells were fixed, permeabilized and used for EGFR immunostaining (Neomarkers, Ab-1) (green, B,F,J,N) and MUC1 (Neomarkers, CT2) (red, A,E,I,M). (A-H') Under serum-starvation conditions (– serum) EGFR localized to the plasma membrane (white arrows), nucleus and cytoplasm. (I-L) In the presence of EGF and MUC1, EGFR is localized within the nucleus (white arrowheads, L'). (M-P') EGF treatment in the absence of MUC1 resulted in EGFR localization to perinuclear regions (asterisk, P'). DAPI, blue nuclei. Scale bars: 20 µm.

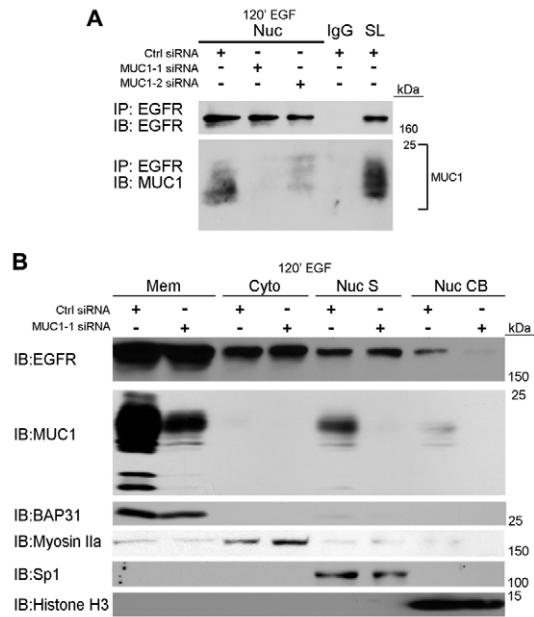


Fig. 3. MUC1-EGFR interaction in the nucleus promotes EGFR-chromatin interaction. (A) MCF10A immortalized breast epithelial cells were treated with control or *MUC1-1* siRNA. Cells were serum starved and treated with EGF (10 ng/ml, 120 minutes at 37°C). The nuclear protein fraction (400 µg) was used for immunoprecipitation (IP) with antibodies against EGFR (Neomarkers, Ab-13) and mouse IgG, and the precipitated protein was separated by SDS-PAGE and immunoblotted (IB) for EGFR (Santa Cruz, 1005) and MUC1 (Neomarker, CT2). SL, straight lysate (non-IP protein, 12 µg). Fraction purity was confirmed by immunoblotting for histone H3 (Santa Cruz, C-16) and myosin IIa (Santa Cruz, D16). Note that straight lysates for this experiment are shown in Fig. 1B. (B) MDA-MB-468 breast cancer cells were fractionated into membrane (Mem), cytosolic (Cyto), nuclear-soluble (Nuc S) and chromatin-bound (Nuc CB) fractions and separated (15 µg) by SDS-PAGE and immunoblotted (IB) for EGFR (Santa Cruz, 1005) and MUC1 (Neomarker, CT2). Fraction purity and loading was determined by immunoblotting for BAP31 (American Bio-Reagents, MA3-002), myosin IIa (Santa Cruz, D16), Sp1 (Santa Cruz, 1C6) and histone H3 (Santa Cruz, C-16). Molecular masses (kDa) are indicated on the side.

Therefore, the presence of MUC1 appears not to alter accumulation of soluble EGFR in the nucleus, but appears to be vital for the interaction of EGFR with DNA.

EGFR colocalizes with phosphorylated RNA polymerase II

Considering the ability of EGFR to interact with chromatin and to directly induce transcription, we next wanted to determine whether MUC1 affected EGFR-mediated transcriptional activation (Lin et al., 2001). A classical marker for transcription initiation is phosphorylated serine-2 RNA polymerase II (pS2-CTD-RNAPolII) (Zhang et al., 2008). Therefore, to visualize EGFR and pS2-CTD-RNAPolII localization, MCF10A cells were transfected with control or *MUC1-1* siRNA and stimulated with 20 ng/ml EGF for 120 minutes. Following EGF treatment, cells were fixed, permeabilized and pS2-CTD-RNAPolII and EGFR were immunolocalized. Under serum starvation conditions, EGFR was mainly observed at the plasma membrane and there was no positive pS2-CTD-RNAPolII staining regardless of MUC1 status (data not shown). By contrast, in the presence of EGF, pS2-CTD-RNAPolII was readily observed in the nucleus in the presence of MUC1 (Fig. 4B). Additionally,

following 120 minutes of EGF treatment in MUC1-expressing cells, there were a significant number of EGFR-positive punctate spots within the nucleus (Fig. 4A) as observed in Fig. 2J. This nuclear EGFR was found colocalized with pS2-CTD-RNAPolII in the presence, but not in the absence of MUC1 (Fig. 4D', compare with 4H). Note that the colocalization indicates that EGFR localizes to transcriptional start sites (Zhang et al., 2008). To confirm that the activity of pS2-CTD-RNAPolII localization was not dependent on MUC1, we also treated cells with 50 ng/ml insulin. We found that insulin treatment for 120 minutes resulted in punctate pS2-CTD-RNAPolII nuclear localization (Fig. 4J, asterisks) and did not affect membrane localization of EGFR (Fig. 4I, arrowhead). These data suggest that MUC1 has a regulatory role in both EGFR trafficking to the nucleus and its interaction with active transcriptional elements.

MUC1 promotes EGFR-mediated cyclin D1 expression

The ability of EGFR to activate transcription of *CCND1* and the importance of cyclin D1 in breast cancer progression has previously been described (Lin et al., 2001). EGFR interacts with the adenine/thymine-rich sequence (ATRS) promoter region of *CCND1* on promoter constructs in vitro, although EGFR has not yet been shown to actively engage the endogenous *CCND1* promoter. Additionally, in an EGFR-dependent tumorigenesis mouse model, the protein expression of cyclin D1 was found to be MUC1 dependent. Therefore, to determine the role of MUC1 in EGFR-induced transcriptional activation of *CCND1*, we used chromatin immunoprecipitation (ChIP) directed against EGFR.

MCF10A cells were treated with *MUC1*-specific siRNA and subsequently stimulated with EGF to promote endocytosis and trafficking of EGFR. The EGFR-DNA complexes were isolated and the ATRS region of the *CCND1* promoter was amplified by PCR to determine whether EGFR association was altered by MUC1. Additionally, we amplified the promoter of the housekeeping gene *GAPDH*, to confirm interaction with RNA Pol II and to determine the purity of the EGFR ChIP. We discovered that EGFR efficiently interacted with the ATRS region of the *CCND1* promoter and this interaction was enhanced in the presence of MUC1 (Fig. 5). This ChIP analysis was performed using extracellular EGFR (Neomarkers, Ab-13) antibody, indicating that the EGFR ectodomain also translocated to the nucleus and engaged the promoter. Additionally, in cells that express MUC1, a ChIP performed against RNA Pol II revealed that RNA Pol II was interacting with the *CCND1* promoter more frequently compared with cells that lacked MUC1 (Fig. 5). We also found that RNA Pol II was associated with the *GAPDH* promoter.

Previously, we demonstrated that the loss of MUC1 expression corresponded to a significant decrease of the interaction of EGFR with the *CCND1* promoter. To determine whether this increased interaction with the *CCND1* promoter translated into increased cyclin D1 protein, we extracted protein lysates from cells treated with control or *MUC1* siRNA. We discovered that the loss of MUC1 resulted in significant loss of cyclin D1 protein expression (Fig. 6A). In addition, the increase was EGF dependent, indicating that although EGFR can be found in the nucleus of serum-starved cells, EGF treatment is necessary to promote cyclin D1 expression. Densitometry analysis of three separate experiments indicated there was a significant 1.4-fold increase in the expression of cyclin D1 (Fig. 6B). These observations demonstrate that EGFR in the nucleus interacts with MUC1 to promote the transcription of *CCND1*.

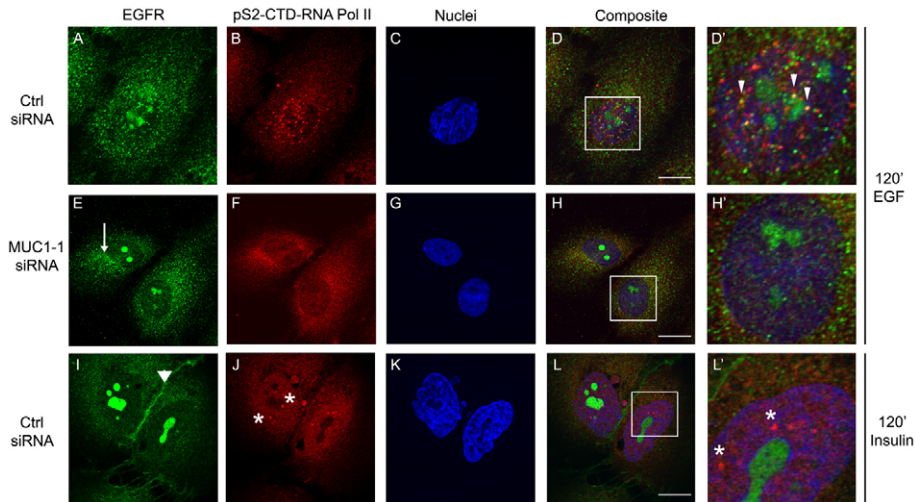


Fig. 4. EGFR colocalizes with phosphorylated RNA polymerase II. MCF10A cells were treated with *MUC1-1* (E-H') or control (Ctrl) siRNA (A-D', I-L') and stimulated with 20 ng/ml EGF for 120 minutes. Following treatment, the cells were fixed, permeabilized and incubated with antibodies against EGFR (Santa Cruz, 1005) (green, A,E,I) and phosphorylated serine-2 RNA polymerase II (pS2-CTD-RNAPolII) (Abcam, H5) (red, B,F,J). Cells were treated with DAPI to visualize nuclei (C,G,K) and composite colocalization of all three images are shown in D,H and L. (A-D) Colocalization (yellow) is seen at the arrowheads (D'). Cytoplasmic EGFR staining is shown by arrow in E. (I-L) Cells were also treated with insulin (50 ng/ml) to illustrate membrane EGFR localization (arrowhead, I) and pS2-CTD-RNA Pol II without EGF treatment (asterisks, L'). DAPI, blue nuclei. Scale bars: 10 μ m.

Discussion

This report identifies a regulatory function of MUC1 on the cellular localization and nuclear activity of EGFR. In breast cancer cells, we observed that both MUC1 and EGFR localize to the nucleus and that MUC1 expression promotes the nuclear accumulation of EGFR regardless of exogenous ligand. Focusing on the nuclear proteins, we were able to efficiently immunoprecipitate EGFR-MUC1 complexes and found that the presence of MUC1 regulated the interaction of EGFR and chromatin. After EGF stimulation in MUC1-expressing cells, we also detected significant colocalization of nuclear EGFR and phosphorylated RNA Pol II. Subsequent examination of chromatin-bound EGFR with an extracellular EGFR antibody revealed that MUC1 expression promoted the interaction of EGFR with the ATRS region of the *CCND1* promoter. The loss of interaction between the *CCND1* promoter and EGFR directly translated to a MUC1-dependent decrease in the expression of cyclin D1.

Previously, MUC1 and EGFR have been reported to interact in a cancer-dependent fashion at the plasma membrane and this interaction inhibited the lysosomal degradation of EGFR (Pochampalli et al., 2007a; Pochampalli et al., 2007b). In addition, overexpression of EGFR leads to altered trafficking of internalized receptor (Liao and Carpenter, 2007). Altered trafficking can include (1) loss of lysosomal targeting, (2) increased recycling to the plasma membrane, and (3) retrograde trafficking. Retrograde

trafficking can occur when transmembrane receptors are endocytosed and returned to the endoplasmic reticulum (ER). Once in the ER, EGFR associates with the Sec61 translocon and is released into the cytosol. EGFR then associates with importin- β 1 via its tripartite NLS and is imported into the nucleus (Lo et al., 2006). In the current report, we demonstrate that MUC1 has a novel regulatory role in EGFR trafficking to the nucleus, by promoting accumulation of nuclear-localized chromatin-bound EGFR. This implies that both EGFR and MUC1 undergo retrograde

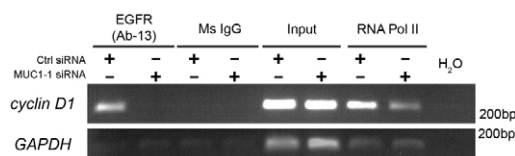


Fig. 5. EGFR interaction with the *CCND1* promoter is MUC1 dependent. MCF10A cells were transfected with either *MUC1-1* or control (Ctrl) siRNA, serum starved, treated with 10 ng/ml EGF, and incubated for 120 minutes. Protein-DNA complexes were collected and chromatin immunoprecipitations were performed against EGFR (Neomarker, AB-13), mouse IgG and RNA Pol II (Millipore, clone CTD4H8). DNA was isolated from the immunoprecipitations and used for PCR against the ATRS region of the *CCND1* promoter and the *GAPDH* promoter.

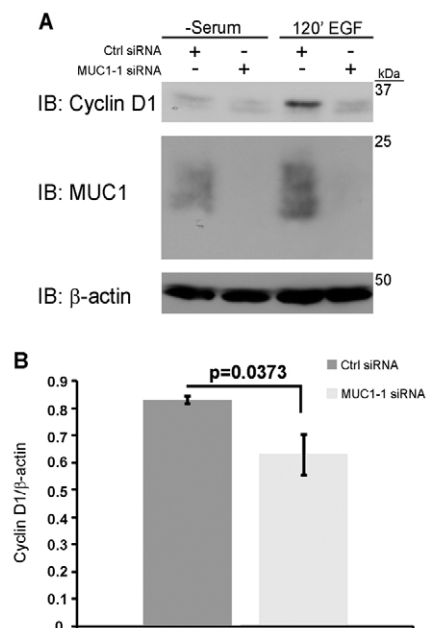


Fig. 6. MUC1 promotes cyclin D1 expression. (A) MCF10A cells were treated with control (Ctrl) or *MUC1-1* siRNA and stimulated with 20 ng/ml EGF for 120 minutes. Cytosolic protein was isolated, analyzed by SDS-PAGE and immunoblotted for cyclin D1 (Cell Signaling, 2922), MUC1 (Neomarker, CT2) and β -actin (Sigma, AC-15). Molecular masses (kDa) are indicated on the right. (B) Relative Cyclin D1 expression levels were determined by densitometry and two-tailed Student's *t*-test was performed based on three experiments.

trafficking and that MUC1 promotes EGFR interaction with chromatin. Additionally, a recent study has demonstrated that MUC1 directly modulates the interaction of a transcription factor with DNA, which results in MUC1-dependent transcriptional activation (Ahmad et al., 2009). Therefore, it is possible that the protein-protein interaction between MUC1 and EGFR alters the conformation of EGFR in a way that allows EGFR to interact with DNA. In addition, MUC1 and EGFR could be serving as transcriptional cofactors, and this will be the subject of future studies.

Interestingly, we found that the nuclear translocation of EGFR was not dependent upon the addition of exogenous ligand. In addition, we found that the ability of MUC1 to influence the EGFR-chromatin interaction required exogenous EGF. We also observed that the ability of EGFR to colocalize with pS2-CTD-RNAPolII and interact with the *CCND1* promoter was dependent on both MUC1 and EGF. Although some reports have found that the nuclear localization of EGFR is dependent on EGF stimulation, others have determined that overexpression of EGFR or autocrine ligand production can drive nuclear accumulation in the absence of exogenous ligand (Liao and Carpenter, 2007; Lin et al., 2001). Our data suggest that both EGF-dependent and -independent mechanisms might be used during nuclear translocation.

Recently, the importance of EGFR kinase activity was examined by using a kinase-dead mutant of EGFR. The authors demonstrated that this kinase-dead EGFR was able to inhibit

autophagic cell death in human cancer cells (Weihua et al., 2008). This study demonstrated EGFR oncogenic action that is independent of its kinase activity. This also suggests that targeting the kinase domain should not be the only focus of EGFR therapeutics. The widely held concept that the tyrosine activity of EGFR confers its role in tumor formation has resulted in several therapeutic approaches that specifically target the EGFR kinase domain. For example, gefitinib was designed to inhibit EGFR dimerization and the resulting receptor activation. These types of therapies were shown to be effective in vitro and in mouse models of tumorigenesis; however, the response rates were not recapitulated in human studies (for a review, see Anders and Carey, 2008). Interestingly, one of the cell lines used in this study (MDA-MB-468) is resistant to gefitinib (Oliveras-Ferraro et al., 2008); the authors discovered that the nuclear accumulation of EGFR was unchanged in MDA-MB-468 cells treated with gefitinib. We have demonstrated that MUC1 alters the ability of EGFR to interact with chromatin in MDA-MB-468 cells, and it is possible that this interaction promotes gefitinib resistance.

Signal-transduction cascades starting at the plasma membrane and using intracellular signaling proteins, such as MAPK, are considered to be the main regulatory mechanism of cyclin D1 expression. Recently, it has been proposed that the expression of cyclin D1 can be regulated through the direct interaction of EGFR and the *CCND1* promoter. Understanding the regulation of cyclin D1 expression is essential to clarifying its role in cancer progression. Aberrant expression of cyclin D1 has been observed

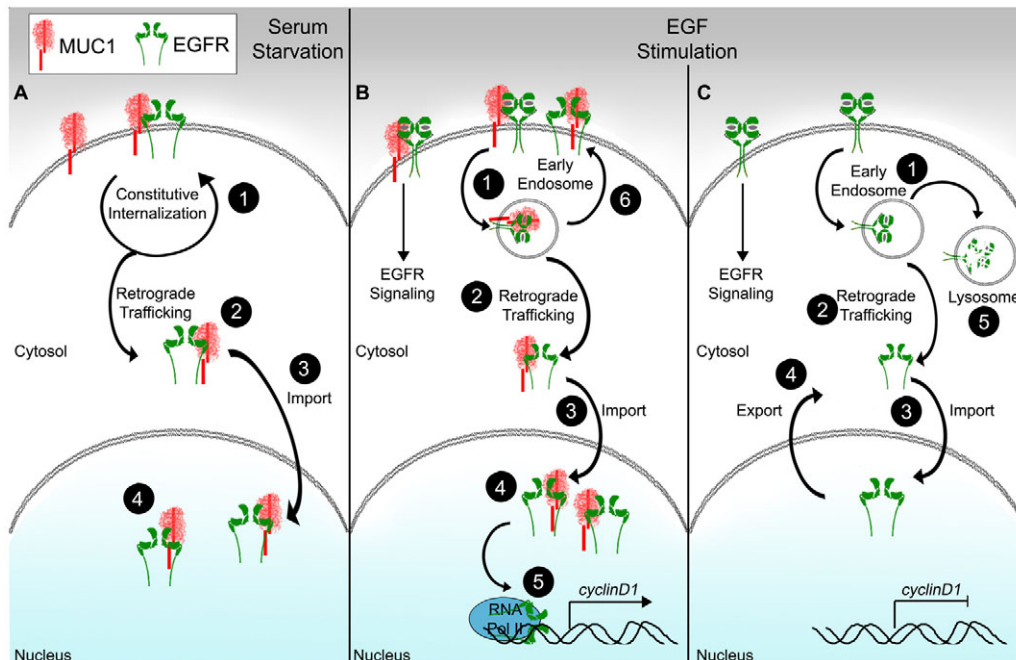


Fig. 7. The role of MUC1 in EGFR nuclear trafficking and function. (A) Absence of EGF. (1) Constitutive internalization of MUC1 to maintain glycosylation also results in EGFR being endocytosed. (2) EGFR and MUC1 both undergo retrograde trafficking through the ER and are released into the cytosol by the Sec61 translocon. (3) Both proteins are able to interact with importin- β 1, which mediates entry in the nucleus. (4) Interaction of MUC1 and EGFR in the nucleus promotes nuclear EGFR accumulation. (B) EGF stimulation in the presence of MUC1. (1) Following EGF treatment, EGFR is rapidly endocytosed in a clathrin-coated vesicle. (2) EGFR and MUC1 undergo retrograde trafficking. (3,4) Both proteins are imported into the nucleus and form a complex. (5) EGFR colocalizes with pRNA Pol II and associates with the *CCND1* promoter to activate transcription. (6) Expression of MUC1 promotes the recycling of EGFR back to the plasma membrane. (C) EGF stimulation in the absence of MUC1. (1) EGF treatment results in EGFR endocytosis. EGFR is either retrograde trafficked (2) or is degraded in the lysosome (5). (3) Cytosolic EGFR is imported into the nucleus and the absence of MUC1 inhibits EGFR nuclear accumulation. (4) EGFR is then exported out of the nucleus.

in 30% of breast cancer, which leads to increased proliferation and genetic instability in both in vivo and in vitro models of breast cancer (Aggarwal et al., 2007; Gillett et al., 1994). Loss of cyclin D1 expression in transformed human cells results in decreased tumorigenicity in nude mice (Kornmann et al., 1998). In addition, the overexpression of cyclin D1 correlates with gefitinib resistance in head and neck squamous carcinoma cells (Kalish et al., 2004). In breast cancer, the overexpression of EGFR and cyclin D1 is not associated with gene amplification, but postulated to be a consequence of an unknown stimulatory mechanism (Gillett et al., 1994). Our data suggest that expression of MUC1 allows EGFR to bind the *CCND1* promoter, which drives tumor progression (Fig. 7).

In conclusion, this report offers a mechanism of transformation as a result of aberrant trafficking and mislocalization of a receptor tyrosine kinase. This newly discovered function of MUC1 in the regulation of EGFR as a transcriptional cofactor sheds light on tumor-dependent alterations in trafficking.

Materials and Methods

Cell culture

MCF10A and MDA-MB-468 cells were obtained from American Type Culture Collection. MCF10A is an immortalized breast epithelial cell line, whereas MDA-MB-468 is a transformed epithelial cell line. MCF10A cells were maintained in RPMI with 10% FBS (Omega Scientific), 1% penicillin-streptomycin (Cellgro), 10 ng/ml cholera toxin (Sigma), 0.5 µg/ml hydrocortisone and 5 ng/ml EGF at 37°C and 5% CO₂. The MDA-MB-468 cells were grown in RPMI with 10% FBS and 1% penicillin-streptomycin at 37°C and 5% CO₂.

Transfection with siRNA

Transfection with siRNA was performed as described (Pochampalli et al., 2007b). The *MUC1*-1 siRNA targets the extracellular domain (3104: 5'-AAGAC-TGATGCCAGTAGCACT-3') (Lan et al., 1990) and the siGENOME SMARTpool (M-004019-02-0020, Dharmacon/ThermoScientific) *MUC1*-2 siRNA targets the 3'UTR and open-reading frame (1: 5'-ACCAAGAGCUGCAGAGAGA-3'; 2: 5'-GAUCGUAGCCCUAUGAGA-3'; 3: 5'-GCCGAAAGAACUACGGGCA-3' and 4: 5'-CCACCAAUUCUCGGACAC-3'). Additionally, the control siRNA has no known mammalian gene targets (5'-AATTCTCCGAACGTGTCACGT-3') (Qiagen). The transfections were performed with Lipofectamine 2000 (Invitrogen) as per the manufacturer's specifications. Cells were transfected, incubated for a specific time (MDA-MB-468, 2 days and MCF10A, 3 days) and used for experiments.

Growth factor treatment

Before growth factor treatment, cells were serum-starved overnight. Cells that were to be used to study the effect of EGF treatment were incubated with 10 ng/ml or 20 ng/ml receptor-grade EGF (Invitrogen) for 10 minutes on ice. Free EGF was then washed twice with PBS and serum-free medium was added. Cells were then incubated at 37°C in serum-free medium for the required time period before being lysed and fractionated.

Differential detergent fractionation

The differential detergent-fractionation protocol followed was modified from a published method (Ramsby et al., 1994). Before lysis, cells were washed, collected and resuspended in cold PBS. The cell suspension was then centrifuged for 2 minutes at 200 *g*, and the PBS was removed. To extract the cytoplasm, cells obtained from the previous step were resuspended in 300 µl digitonin lysis buffer [0.01% digitonin, 100 mM NaCl, 300 mM sucrose, 10 mM PIPES (pH 6.8), 3 mM MgCl₂, 5 mM EDTA, 2.0 mM sodium orthovanadate, 50.0 µM ammonium molybdate and 10.0 mM sodium fluoride and Complete protease inhibitors (Roche)] and incubated on ice for 5 minutes. The insoluble material was then pelleted for 10 minutes at 400 *g* and the supernatant was stored as the cytosolic fraction.

The pellet obtained from the cytoplasmic centrifugation was resuspended in 300 µl Triton X-100 lysis buffer [0.5% Triton X-100, 100 mM NaCl, 300 mM sucrose, 10 mM PIPES (pH 7.4), 3 mM MgCl₂, 3 mM EDTA, 2 mM sodium orthovanadate, 50 µM ammonium molybdate and 10 mM sodium fluoride and Complete protease inhibitors (Roche)] and incubated on ice for 5 minutes. Cells were then subjected to centrifugation for 10 minutes at 5000 *g* and the supernatant was stored as the membrane fraction.

The pellet obtained from the membrane centrifugation was resuspended in 500 µl Tween 40/DOC lysis buffer [1% Tween-40, 10 mM NaCl, 0.5% deoxycholate, 10 mM PIPES (pH 7.4), 1 mM MgCl₂, 2 mM sodium orthovanadate, 50 µM ammonium molybdate and 10 mM sodium fluoride and Complete protease inhibitors (Roche)]. The pellets were then homogenized 20 times with a B-dounce and sonicated three

times for 10 seconds each. This was followed by centrifugation for 10 minutes at 7000 *g* and the supernatant was removed as the nuclear fraction.

Chromatin-bound protein isolation

Chromatin-bound nuclear protein fractions were extracted per the manufacturer's protocol using the Subcellular Protein Fractionation Kit from Thermo Scientific. Note that nuclear extraction buffer is considered 'high-salt' by Thermo Scientific. Next, the soluble nuclear fraction and the chromatin-bound protein fraction were isolated by treating the DNA-protein complexes with Micrococcal nuclease, which digested the DNA thereby releasing the protein into the supernatant. Protein concentrations for all fractions were determined by Bicinchoninic Acid assay (Thermo Scientific) and all of the samples were stored at -80°C until use.

High-salt nuclear extraction

Protein was extracted as described (Lin et al., 2001). Cells were collected in non-nuclear extraction buffer [20 mM HEPES, pH 7.0, 10 mM KCl, 2 mM MgCl₂, 0.5% NP40, 2 mM sodium orthovanadate, 50 µM ammonium molybdate and 10 mM sodium fluoride and Complete protease inhibitors (Roche)] and homogenized in a Dounce homogenizer 30 times. Lysates were centrifuged for 5 minutes at 1500 *g* and supernatant was removed. The pellet (nuclei) was washed three times with non-nuclear extraction buffer, resuspended in high salt nuclear extraction buffer [500 mM NaCl, 20 mM HEPES, pH 7.0, 10 mM KCl, 2 mM MgCl₂, 0.5% NP40, 2 mM sodium orthovanadate, 50 µM ammonium molybdate and 10 mM sodium fluoride and Complete protease inhibitors (Roche)] and incubated at 4°C for 10 minutes. Suspension was centrifuged for 10 minutes at 15,000 *g* and supernatant was collected as the nuclear fraction.

Immunoprecipitation and immunoblotting

Protein was treated as described (Pochampalli et al., 2007b). Nuclear protein was isolated via differential detergent fractionation and the protein concentration was assessed by a Bicinchoninic Acid assay. Protein (400 µg) was incubated in TNEN buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8.0, 0.5% Igepal CA630, 2.0 mM sodium orthovanadate, 50 µM ammonium molybdate, 10 mM sodium fluoride, and Complete protease inhibitors) and primary antibody (EGFR, Ab-13, Neomarkers, 2 µg) or mouse IgG for 1.5 hours at 4°C. The protein-TNEN-antibody mixture was tumbled at 4°C with agarose-G beads (Molecular Probes, Invitrogen) for 4 hours. The precipitations were washed three times with TNEN buffer and then incubated with 2% SDS-PAGE buffer. Immunoprecipitations were separated by SDS-PAGE and transferred to PVDF membrane (Millipore, Billerica, MA). The membrane was blocked in either a 3% milk in PBS/0.1% Tween solution or a 3% BSA in TBS/0.1% Tween and then used for immunoblotting. Proteins on the membrane were treated with Super Signal Chemiluminescent Substrate (Pierce), visualized on Imagemetech-B film (American X-ray, Louisville, TN) and developed with a Konica SRX-101C.

Antibodies

The EGFR (1005), myosin IIa (D16), insulin growth factor-receptor 1β (IGF-R1β, C-20), early endosome antigen 1 (EEA1, H-300), Sp1 (1C6) and histone H3 (C-16) antibodies were purchased from Santa Cruz Biotechnologies. MUC1 (CT-2), EGFR (Ab-13), and EGFR (Ab-1) were purchased from Neomarkers/Thermo Scientific. Cyclin D1 (2922) was purchased from Cell Signaling. BAP31 (MA3-002) was purchased from Affinity Bioreagents. Phosphorylated serine-2 RNA polymerase II was purchased from Abcam (H-5). Secondary antibodies (Alexa Fluor 488 anti-mouse, Alexa Fluor 488 anti-rabbit and Alexa Fluor 594 anti-mouse) for immunofluorescence were purchased from Molecular Probes with the exception of anti-hamster Texas red antibody, which was from Jackson Laboratories.

Immunofluorescence

Cells were grown on coverslips, treated with siRNA and incubated with EGF, as described above. Cells were fixed with a 4% paraformaldehyde-PBS solution and permeabilized with 0.5% Triton X-100, 0.02% Na₂S₂O₄ in PBS. Following fixation, cells were blocked with 20% fetal bovine serum (FBS) and 0.02% Na₂S₂O₄ in PBS and incubated at 4°C for 18 hours with primary antibody (1:100) in 10% FBS, 0.02% Na₂S₂O₄ in PBS (stock buffer). Secondary antibody (1:200) was incubated at room temperature for 45 minutes in stock buffer. The slides were visualized with a Zeiss Axiovert 200 confocal microscope (Molecular and Cellular Biology Microscopy Facility, University of Arizona), images were captured using a Zeiss AxioCam and analyzed with the Zeiss Meta software.

Statistical analysis

Statistic analysis was performed using the STATA 10 program.

Densitometry

Densitometry measurements were carried out using ImageJ (NIH).

ChIP

Epithelial cells were grown in 100 mm dishes and treated with *MUC1* or control siRNA. Cells were exposed to EGF treatment and allowed to incubate for 120 minutes. Cells were then incubated with 1% paraformaldehyde in PBS for 10

minutes at room temperature and then treated 0.125 M glycine for 5 minutes at room temperature. Cells were washed with PBS, resuspended in PBS with 2 mM sodium orthovanadate, 50 μ M ammonium molybdate, 10 mM sodium fluoride and Complete protease inhibitors, and counted. Cells were then snap frozen and kept at -80°C . These cells were subjected to chromatin immunoprecipitation based on the Millipore EZ-ChIP protocol (17-371). Briefly, cells were lysed and the protein or DNA lysates were sonicated (five 10-second bursts) and pre-cleared with DNA blocked agarose-G beads (16-201, Millipore) for 30 minutes at 4°C . The pre-cleared lysates were incubated overnight at 4°C with antibody against EGFR (Neomarkers, Ab-13), Mouse IgG and RNA Pol II (5 μ g, Upstate, clone CTD4H8). The antibody-protein-DNA complexes were isolated by incubating agarose-G beads for 60 minutes at 4°C . The agarose-G-bead-antibody-protein-DNA complexes were washed several times and the crosslink was reversed by incubating with NaCl overnight at 65°C . DNA was treated with RNaseA and proteinase K, and isolated with a Qiagen spin column. DNA was used for PCR to amplify the ATRS region of *CCND1* (F, 5'-TGG-TCTTGTCAGGAGAG-3' and R, 5'-GATGGTGGCCAGCATTC-3') and *GAPDH* (F, 5'-TACTAGCGGTTTACGGGCG-3' and R, 5'-TCGAACAGG-AGGAGCAGAGAGCGA-3'). The *CCND1* PCR was performed for 35 cycles at 98°C for 10 seconds, 65.3°C for 10 seconds, and 72°C for 25 seconds. The *GAPDH* PCR was performed for 32 cycles at 98°C for 10 seconds, 62°C for 10 seconds, and 72°C for 25 seconds. The PCR products were analyzed on a 2% agarose gel and visualized using a Kodak Gel Logic 2000.

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